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EFFECT OF DIBENAMINE ON PROGESTERONE-INDUCED AND "SPONTANEOUS" OVULATION IN THE HEN[1]

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HUSTON and Nalbandov (1953) showed that the presence of a foreign body, usually a thread, in the oviduct of a laying hen inhibits ovulation for as long as 20 days, apparently without altering the rate of hypophyseal function insofar as FSH secretion is concerned. This was confirmed by van Tienhoven (1953). It was thought that a neuro-humoral link existed between the oviduct and hypophysis which could prevent the release of ovulatory quantities of luteinizing hormone (LH) from the pituitary gland. To test this assumption as well as to get additional information on the mechanism of pituitary gland function in the fowl, the experiments discussed here were done. Because Dibenamine is known to be able to block the release of the LH in rats and rabbits (Markee *et al.*, 1952), it was decided to investigate its effect in the hen.

MATERIALS AND METHODS

The hens used were of the S. C. White Leghorn and Columbian breeds. Only hens laying 3–4 eggs per clutch, and with an interval between clutches of not more than 1 day, were used. The birds were kept in individual laying cages and received a standard ration and water *ad libitum*. Natural day-night conditions were maintained during the experiments.

For technical reasons (facility in timing, etc.) it was thought best to perform all the experiments on the first egg of the clutch to be ovulated. To determine accurately whether ovulation had taken place all hens were palpated routinely every morning be-

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[1] Part of the data reported is taken from a thesis presented to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the Ph.D. degree.

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tween 7:00 and 8:00 A.M. and, if no egg was detected, palpation was repeated at about 2:00 P.M. of the same day. If the hen was to be used in an experiment the palpation was done again at 9:00 P.M. as an additional safeguard of the correctness of the diagnosis that she was in the non-ovulatory interval between two successive clutches. All injections were made into the wing vein on the day of lay of the terminal egg of the clutch when normal ovulation would not be expected to occur. Thus any ovulation could be ascribed, with a reasonable degree of certainty, to the treatment given. Oviposition was checked between 8:00 A.M. and 9:00 P.M.

Before a bird was placed on the experiment, palpations were made for a period of 14 days, between 7:00 and 8:00 A.M. This allowed us to determine the time of ovulation of the first egg of a clutch at least 3 times. We believe that the error involved in the determination of ovulation time is not more than 1 hour.

The progesterone[3] used was dissolved at a concentration of 1 mg./cc. of a mixture consisting of 12.1 cc. propyleneglycol, 12.1 cc. alcohol, and 0.1 cc. HCl; this mixture will be referred to as the carrier. Three parts of the progesterone solution were mixed with 2 parts of water just prior to injection.

The Dibenamine[3] (N, N-dibenzyl-β-chloroethylamine) was dissolved at a concentration of 50 mg./cc. in the carrier. When Dibenamine was given in combination with progesterone, the stated amounts per cc. of both substances were dissolved in the carrier. Three parts of the solution were mixed with 2 parts of water prior to injection.

Atropine sulfate was dissolved in water at a concentration of 75 mg./cc. and 2 parts of this solution were mixed with 3 parts of the progesterone solution or 3 parts of the carrier prior to injection.

EXPERIMENTS AND RESULTS

Effect of Dibenamine and Atropine on LH Release

To control the time of LH release to the greatest possible degree, advantage was taken of the known ability of progesterone to induce LH release and ovulation in the fowl about 7–9 hours after the progesterone administration (Fraps and Dury, 1943). To test the effects of Dibenamine and atropine on such progesterone-induced ovulations, the following experiment was carried out.

Forty-eight hens were assigned at random to 3 treatments. The 3 treatments were: intravenous injection of progesterone (0.6 mg./kg.), progesterone at the same level plus Dibenamine (30 mg./kg.), and progesterone at the same level plus atropine (30 mg./kg.).

Dibenamine administration, although fatal in 1 case, did not cause violent reactions in the surviving birds. It resulted in constriction of the pupils, flapping of the wings for about 2 minutes and slight shaking of the head. No after effects, as observed by Sawyer, Markee and Hollinshead (1947) in rabbits, were noticed.

Atropine injection caused gasping for air, dilation of the pupils, and in

[3] The authors are indebted to Dr. R. E. Waterman, Schering Corp., Bloomfield, N. J, for the progesterone, and to Dr. Paul A. Mattis, Smith, Kline, & French Laboratories, Philadelphia, for the Dibenamine.

1 case, in which ovulation was blocked, contractions of the legs and wings occurred. However, after 5 minutes the bird recovered and seemed normal within 30 minutes.

All injections were made at 4:00 P.M. on the day of lay of the terminal egg of the clutch. Progesterone, because it induces ovulation about 7–9 hours after it sinjection, should cause ovulation to take place between 11:00 P.M. and 1:00 A.M. The time for normal ovulation in our hens was about 4:00 A.M. This was determined from palpation records kept previous to the experiment and agrees well with the ovulation time found by Fraps and Case (1953). In 3 cases ovulations occurred shortly after injection, indicating that we had misjudged the termination of the clutch.

Prematurity of the egg was judged by palpation the morning after injection. An egg in the isthmus or a non-plumped membranous egg in the

TABLE 1. EFFECT OF DIBENAMINE AND ATROPINE ON THE BLOCKADE OF PROGESTERONE-INDUCED OVULATIONS IN LAYING HENS

Treatment	Birds inject.	Premature ovulations		Failed premature ovulations		Failed to ovulate	
	no.	no.	%	no.	%	no.	%
Progesterone	14	11	78.5	3	21.5	0	0
Prog. + Dibenamine	14*	1	7.7	12	92.3	10	76.9
Prog. + Atropine	17	11	64.7	6	35.3	5	29.4

* One bird died following Dibenamine injection.

uterus was considered to have been ovulated about 4 hours previously (not a premature ovulation), while a plumped egg in the uterus was considered to have been ovulated at least 8 hours previously (premature).

The results of this experiment are given in Table 1. The data when analyzed statistically for success or failure of blockade of the premature, progesterone-induced, ovulation, showed that the difference between the atropine-treated and the control group was not significant. However, the Dibenamine-treated group was highly significantly different ($P < 0.01$) from the controls as well as from the atropine-treated group.

Of the 10 birds failing to ovulate in the progesterone-Dibenamine group, 5 returned to normal production within 2 days after injection, 2 in 3 days, 1 in 4 days, 1 in 6 days and 1 in 15 days. Of the 5 birds that did not ovulate on the progesterone-atropine treatment, all returned to normal production within 3 days. The fact that the combs remained normal in size and color, and that all but 1 bird resumed production within 1 week, was taken as indirect evidence that the ovaries remained normal and that, except for the one bird not returning to production within a week, no general atresia of the large follicles occurred. Unpublished data (van Tienhoven, 1953) showed that in 3 out of 5 birds no atresia occurred, one hen's largest

and second largest follicle were atretic while one bird showed complete atresia of all follicles. More data have to be collected on this particular phase in order to interpret the significance of this phenomenon.

Time Interval between LH Release and Ovulation

The first experiment showed that Dibenamine was a satisfactory agent for blocking progesterone-induced ovulation, but that atropine was not satisfactory at the dose used. Therefore, Dibenamine was used in a series of experiments to estimate the time interval between the stimulus for LH release and ovulation. In these experiments also the first egg of the clutch was used.

Because of the relatively small number of birds available, 3 experiments were carried out using the same birds. Between the first and second of this series of experiments 3–4 weeks elapsed, and between the second and third there was a time interval of 1 week. In all cases only those birds were used which showed a normal laying cycle of no less than 4 eggs per clutch. The birds were randomized separately for each experiment.

As the statistical analysis showed no significant difference in the results of the 3 experiments, the data were pooled and the results are reported as one experiment. In this design the birds were injected with 30 mg./kg. of Dibenamine at 4, 6, 8, 10, 12, and 14 hours before expected ovulation of the first egg of the clutch. One control group was given the carrier without Dibenamine 8 hours before expected ovulation. Palpation records, obtained before the experiment started, showed that the time of expected ovulation of the first egg of the clutch was about 4:00 A.M., and all birds were injected and palpated in accordance with this assumption.

In this experiment 2 birds died of Dibenamine administration. The results obtained with respect to blockade of ovulation are given in Table 2.

On statistical analysis it was found that the incidence of ovulation in the treated groups showed a heterogenous distribution ($\chi^2 = 24.82$, $P < 0.001$). It was also found that the deviations from a straight regression line on time, using apprporiate statistical weights, gave a χ^2 of 15.74 ($P < 0.1$). Because the 3 delayed ovulations, observed at the 12-hour administration, suggested that a peculiar phenomenon might have occurred causing the large deviation at 12 hours, the statistical process was repeated omitting these three observations. This resulted in a χ^2 of 10.65 ($P = 0.03$). Apparently, the large deviation was not due to the fact that the delayed ovulations had been counted as not blocked.

When the 12-hour point was not used in fitting the line, the χ^2 decreased to a value of 1.28, indicating that the failure to obtain a good fit to the whole data was due to the large deviation of the "12-hour" observations.

The validity of this experiment depends on the accuracy with which the

TABLE 2. INCIDENCE OF BLOCKADE OF OVULATION WITH DIBENAMINE ADMINISTERED INTRAVENOUSLY AT VARIOUS INTERVALS BEFORE EXPECTED OVULATION

Dibenamine dose	Time before expected ovulation	No. of birds	No. of birds ovulated	No. of birds failing to ovulate	Per cent blocked
mg./kg.	hours				
30	4	7	7	0	0
30	6	12	9	3	25.0
30	8	12	6	6	50.0
30	10	14	6	8	57.1
30	12	16	13*	3	18.7
30	14	16	2	14	87.5
—	8	13	12	1†	—

* Three birds ovulated about 4 hours after expected time of ovulation.
† This bird ovulated 24 hours after the expected time. This was not considered as a "blocked" ovulation.

time of ovulation could be predicted. Palpation records kept before birds were placed on the experiments give a good indication of expected ovulation time. However, variations between first ovulation times might occur for reasons unknown to us. The fact that ovulations which were not blocked, occurred at the expected time was taken as evidence for the accuracy of the prediction.

DISCUSSION

In general, the hen's reproductive cycle has been thought to be under the control of hypophyseal and ovarian hormones. There is an increasing amount of evidence indicating that factors other than hormones are involved in the control of ovulation.

Huston and Nalbandov (1953) suggest that an irritant in the oviduct inhibits the sudden (?) release of LH from the anterior pituitary gland. It is possible that this effect is an artifact, and that it has no physiological significance. However, the quantitative differences in efficiency of inhibition of LH release, between the upper magnum and isthmus shown by van Tienhoven (1953), suggest that it has a physiological importance. Further investigation is needed to determine whether the presence of an egg in the oviduct has the same control over hypophyseal function as does a thread.

It is possible that in the hen progesterone is the hormone responsible for LH release. That exogenous progesterone has this effect has been shown by Fraps and Dury (1943) and was confirmed by us. It was further found that progesterone-induced ovulations can be blocked by Dibenamine, which shows that this hormone in the hen acts on the anterior pituitary gland via a neural mechanism. Using different techniques a similar mechanism was suggested by Fraps and Case (1953). That Dibenamine acts in the manner indicated, rather than by impairing the ability of follicles to ovulate, is supported by the finding that out of 34 hens, in which ovulation

was blocked by Dibenamine, 8 ovulated one day, 8 two days and 5 three days after expected ovulation. Furthermore, it was found that Dibenamine injected 4 hours before ovulation did not interfere with the release of the first ovum at the expected time. Neither did it modify the subsequent laying performance of hens treated at that time.

The similarity of results obtained in the hen and in mammals suggests that the inhibition of spontaneous ovulation in the hen probably acts through a mechanism similar to the one postulated in mammals, namely by blocking the effect of an adrenergic mediator. To determine whether this is true experiments are planned to investigate the effect of epinephrine on ovulation in the hen.

Dibenamine was very effective at blocking ovulations when injected 14 hours before expected follicle rupture. The effectiveness of this blockade decreased progressively from 14 to 4 hours except at the 12-hour point where there was a significant deviation which remains unexplained. The fact that 3 delayed ovulations took place at that point, and none at the others, suggests that a delicate balance exists at this particular time between the effect of the stimulus and the blocking effect of Dibenamine. Because of the unknown nature of the stimulus, we have not been able to determine the cause of this 12-hour effect. The fact that the most effective blockade occurs at 14 hours suggests that the stimulus for LH release and hence ovulation takes place about 14 hours prior to follicle rupture. Apparently, LH release and thus ovulation takes place about 8 hours prior to ovulation in half of the birds, while in some it occurs as much as 14 hours before expected ovulation. Under our experimental conditions stimulation would take place in part of the birds at approximately 2:00 P.M. This interval was estimated by Fraps, Riley, and Olsen (1942); Fraps, Olsen, and Neher (1942); Fraps and Dury (1943); and Rothchild and Fraps (1949), to be 8 hours, disagreeing with our results. The reason for this difference is unknown. It remains to be seen which estimate is more correct, although the Dibenamine technique used here seems more accurate because it does not involve hypophysectomy, anesthesia, surgery, exogenous LH administration to non-hypophysectomized hens, or progestrone administration, which might sensitize the ovary to endogenous LH in addition to its ability to cause LH release.

The asssumed time interval of 14 hours between stimulation of the hypophysis and LH release seems to show a discrepancy with the fact that progesterone, when given later than the time of stimulation, causes premature ovulation. It has been shown by Rothchild and Fraps (1949) that the hypophysis of the hen has to remain *in situ* for 4 hours after progesterone injection, in order to obtain a maximum normal ovulating response. Everett and Sawyer (1953) presented experimental evidence indicating

that the duration of the stimulus of the rat hypophysis for normal ovulation was 20–35 minutes. In the same publication, these authors suggest stimulation of the anterior pituitary gland and the release of LH may take place concurrently.

The above cited data of Rothchild and Fraps (1949) suggest that the duration of release of LH in the hen may be considerably longer than in the rat. Whether stimulation and release take place concurrently is under investigation.

The effect of progesterone, inducing premature ovulation, could be due to the lowering of the threshold for the stimulus causing a larger quantity of LH to be released in a short time. In this case, the stimulation would not be an all-or-none effect but would have the nature of a graded response. If this assumption is correct, the effect of Dibenamine on progesterone-induced ovulation would be due to the interruption of the stimulation of the hypophysis rather than to the blocking of the progesterone effect as such.

The fact that atropine did not block progesterone-induced ovulation in our experiments might well be due to an inadequate dose of atropine. This problem is still under investigation.

SUMMARY AND CONCLUSIONS

1. Using the first egg of a clutch in regularly laying hens, it was shown that Dibenamine is capable of blocking spontaneous as well as progesterone-induced ovulations.

2. It was shown that a neural mechanism is involved in spontaneous as well as in progesterone-induced ovulations.

3. Dibenamine blocks spontaneous ovulations most effectively when injected 14 hours before ovulation and is progressively less effective at 10, 8, 6, and 4 hours. At 12 hours, an unexplained low rate of blocking was observed.

4. The results suggest that in the hen LH is released 8–14 hours before ovulation in the majority of the birds observed.

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THE EFFECTS OF INDUCED HYPERTHYROIDISM AND VITAMIN B_{12} ON THE SYNTHESIS OF SOME PHOSPHATE FRACTIONS IN THE RAT LIVER

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THE influence of thyrotoxic agents on the turnover of labelled phosphate in various organic compounds of the adult rat liver has been demonstrated by several workers (Flock, Bollman and Berkson, 1948; Fraenkel-Conrat and Li, 1949; Venkataraman, *et al.*, 1950). These *in vivo* studies, in general, indicate that the thyrotoxic animals have a tendency toward an increased turnover of phosphates in organic linkage. Similar observations have been recorded with intact tissue slices of thyrotoxic rats under conditions of aerobic glycolysis (Goldstein, 1952).

Recently, vitamin B_{12} has been shown to counteract the retardation of growth in young rats which results from administering toxic amounts of thyroid powder or thyroprotein (Betheil and Lardy, 1948; Nichol, Dietrich, Cravens and Elvehjem, 1949; Emerson, 1949; Meites, 1950). Other B vitamins which prevent weight loss in the hyperthyroid mature rat (Drill, Overman and Leathem, 1943) are ineffective in this respect in the immature hyperthyroid rat (Ershoff, 1947). Oginsky (1950) has shown that the *in vitro* synthesis of methionine by livers of hyperthyroid rats was stimulated when vitamin B_{12} was fed. Rupp *et al.* (1951) found that the loss of nitrogen caused by the catabolic action of thyroxine was reduced significantly by the vitamin.

These facts prompted a study of the effects of hyperthyroidism and vitamin B_{12} on the rate of incorporation of radiophosphorus into some of the organic phosphate fractions of the immature rat liver.

EXPERIMENTAL

Animals, Care and Treatment

Weanling male rats of the Sprague-Dawley strain, within a weight range of 38–50 gm., were employed throughout. The animals were housed in groups of two or three in cages

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with raised screen floors. The basal ration consisted of cornmeal 45.75%, soybean meal[1] 45.75%, salts (USP XIII) 2%, corn oil 5%, and L-cystine 0.3%.

The vitamin composition of the ration was the same as that described by D'Iorio and Plaut (1952). All animals received 3 drops of oil percomorphum diluted 1:4 with corn oil, weekly. Food and water was supplied *ad libitum*. Table 1 presents information concerning the endocrine and vitamin treatment, average initial and final body weight and size of the various groups of animals.

On the last day of the experiment, after a previous fasting of 10–12 hours, the animals were injected intraperitoneally with approximately 10 μc. of radioactive disodium phosphate,[2] per 100 gm. body weight, in normal saline, pH 7.4. The total amount of phosphate administered was physiologically negligible, being less than 0.2 mgm. Six hours later, the animals were anesthetised with nembutal, 6 mgm. per 100 gm. body weight, and exsanguinated. The livers were quickly removed, blotted to remove adhering blood and frozen in dry ice-acetone bath. The tissues were stored in deep freeze ($-20°$ to $-30°$) until required for processing. Because the entire liver could not be fractionated, duplicate specimens were routinely analyzed.

Chemical Methods

The fractionation of the tissue for total acid-soluble, phospholipid, pentosenucleic acid (PNA), desoxypentosenucleic acid (DNA) and phosphoprotein[3] (PP) phosphorus was carried out by the method of Schmidt and Thannhauser (1945) as adapted to isotope studies by Johnson and Albert (1953) and Albert, Johnson and Choan (1951). The alcohol-ether extract of the phospholipid was concentrated at room temperature *in vacuo* to dryness. The residue was extracted into chloroform (Artom, 1941). Aliquots of the chloroform solution were used for determination of phosphorus content. Before radioactive measurement, the chloroform solution of the phospholipid was shaken with powdered sodium phosphate (Artom and Cornatzer, 1947). Phosphorus (P^{31}) content of the various fractions was determined by the method of Fiske and Subbarow (1935) as modified by LaPage and Umbriet (1945).

Radioactive measurements

Aliquots of samples were transferred to stainless steel cupped planchets greased inside to prevent creeping of samples on the walls. To ensure uniform spreading of the samples, 2 drops of 1% solution of aerosol O.T. (a synthetic detergent) and 0.5 cc. of 1% disodium hydrogen phosphate were added. In the case of phospholipid, aliquots of chloroform extracts were placed on lens paper mounted on nickel plated cupped planchets. All samples were evaporated to dryness at room temperature under a fan and were counted with a thin end window Geiger-Muller counter. The counting rate of the samples was generally six times over the background, except in the case of DNA. Geiger counts of DNA samples that did not exceed the background by 10 counts were not considered significant, 10 counts being approximately 3 times the standard error of the background. Duplicate determinations or radioactivity were carried out and agreed within 5 per cent.

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[1] Solvent extracted, 44% protein.

[2] The radioactive P used in these experiments was furnished by the Oak Ridge National Laboratory, Oak Ridge, Tennessee, on allocation from the U. S. Atomic Energy Commission.

[3] The non-nucleic acid lipid free protein in combination with phosphate will be referred to as phosphoprotein.

TABLE 1. DESCRIPTION OF TREATMENT OF EXPERIMENTAL GROUPS AND ALTERATION IN BODY WEIGHTS

Group No.	Treatment	No. of rats	Average body weight in gm.	
			Initial	After 4 weeks
I	Basal ration for 4 weeks +2 μg. vitamin B_{12} per week.*	16	47	181
II	Basal ration +0.2% iodinated casein† for 4 weeks.	14	41	120
III	Basal ration +0.2% iodinated casein for 4 weeks; injected 2 μg. vitamin B_{12} per day in the last 2 weeks.	10	43	168

* All B_{12} supplementation was by intraperitoneal injection.
† Protomone, cerophyll Laboratories Inc.

RESULTS

The data in Table 1 shows the characteristic influence of vitamin B_{12} on the weight response of hyperthyroid rats.

The results of P^{32} incorporation into various liver phosphorus fractions together with their phosphorus content are summarised in Table 2. No attempt has been made to fractionate the total acid-soluble phosphorus. Certain marked changes both in the P^{32} and P^{31} values of the fraction in the three groups warrant remarks in passing. While the phosphorus content of groups I and II are similar, administration of vitamin B_{12} to thyrotoxic rats leads to a significant reduction (17%, $P < 0.01$). Comparison of the specific activities reveal a diminished (14%, $P < 0.01$) incorporation of radiophosphorus by the hyperthyroid rat. Supplementation with vitamin B_{12} leads to a partial restoration of the specific activity value, not markedly different ($P > 0.02$) from the control figure. Greenberg, Fraenkel-Conrat, and Glendening (1947) observed that hyperthyroidism in the rat caused a decrease in the P^{32} concentration of serum, acid-soluble fraction of liver and increased P^{32} in the acid-soluble and labile phosphate fractions of the muscle, 110 minutes after administering the tracer. This fact suggested that thyroid activity may influence the rate of transfer of P^{32} across the cell membrane. In the present report we have no data concerning the concentration of P^{32} in serum. Studies of Flock, Bollman, and Berkson (1948) on the time-course of removal of tracer phosphate from the plasma of normal and thyroxine treated rats reveal that the differences in the rates of transfer are pronounced at early intervals and narrow down with time. Therefore, under the conditions of the present experiment, where isotopic equilibrium can be considered to have been established between the plasma and interstital fluid, the specific activities of the fraction in the three groups may reflect on the relative rates of incorporation of P^{32}.

In the present study, the radioactivity values of liver phospholipid,

TABLE 2. INFLUENCE OF HYPERTHYROIDISM AND VITAMIN B_{12} SUPPLEMENTATION ON THE INCORPORATION OF RADIOPHOSPHORUS INTO SOME PHOSPHATE FRACTIONS OF THE IMMATURE RAT LIVER*

Phosphorus fraction	Control (Group I)			Hyperthyroid (Group II)			Hyperthyroid + B_{12} (Group III)		
	P^{31}	s.a. ×100	r.s.a. ×100	P^{31}	s.a. ×100	r.s.a. ×100	P^{31}	s.a. ×100	r.s.a. ×100
	mg.			mg.			mg.		
Total acid-soluble	1.08 ± 0.01	71.1 ± 1.4	—	1.03 ± 0.03	61.5 ± 1.0	—	0.86 ± 0.02	66.3 ± 2.0	—
Phospholipid	0.84 ± 0.04	33.9 ± 1.9	47.7 ± 2.6	0.76 ± 0.04	36.8 ± 2.3	59.8 ± 2.5	0.87 ± 0.05	38.8 ± 2.3	58.6 ± 2.1
Pentosenucleic acid	0.90 ± 0.02	7.1 ± 0.4	10.2 ± 0.5	0.94 ± 0.02	11.6 ± 0.8	17.8 ± 1.3	0.90 ± 0.03	8.91 ± 0.7	13.6 ± 0.7
Desoxypentosenucleic acid	0.25 ± 0.05	1.25 ± 0.05	1.76 ± 0.15	0.27 ± .05	1.12 ± 0.06	1.83 ± 0.10	0.24 ± 0.03	1.20 ± 0.06	1.80 ± 0.20
Phosphoprotein	0.123 ± 0.005	27.0 ± 0.9	38.2 ± 1.6	0.120 ± 0.004	24.0 ± 0.81	39.2 ± 2.0	0.110 ± 0.007	25.2 ± 0.7	37.8 ± 1.8

* The values include the standard error of the mean. The phosphorus (P^{31}) content of total acid-soluble, phospholipid, PNA, DNA and PP fractions are expressed in mg. per gm. of fresh tissue. Specific activity (s.a.) is the per cent of the injected dose per mg. of phosphorus of the fraction. The relative specific activity (r.s.a.) is the s.a. of the fraction divided by the s.a. of the total acid-soluble phosphate six hours after P^{32} injection. The number of animals in each group is indicated in Table 1.

PNA, DNA, and PP fractions have been expressed as "relative specific activities"; that is the ratio of the specific activity of the fraction to that of total acid-soluble phosphorus. This was considered advisable in order to eliminate variation in the final body weight of animals, possible differences in the absorption of the radioisotope as well as other individual animal variations which could not be controlled. A similar index has been employed in the study of phospholipid synthesis in the intestine (Zilversmit, Chaikoff, Entenman, 1948). In regard to nucleic acid syntheis, the validity of such an index appears to be justified in the light of the experiments of Tyner, Heidelberger and LaPage (1953) on the pathways of incorporation of radiophosphorus into nucleic acid nucleotides in the rat liver. Further, the observation of Johnson and Albert (1953) of a correlation between the P^{32} uptake of acid-soluble and phosphoprotein fractions is suggestive of a metabolic link between them.

It is apparent from Table 2 that there is no change in the P^{31} content of the phospholipids in the three groups, although the values are lower than those reported by Flock, Bollman and Berkson (1948). The specific activities of the liver phospholipid of the control and experimental animals are not significantly different from one another. But the relative activities indicate an increased (25%, $P < 0.01$) rate of synthesis of liver phospholipid in groups II and III. The increased synthesis of liver phospholipid in hyperthyroid animals confirms the findings of Flock and coworkers (1948). There is growing evidence to indicate that the turnover rate of liver phospholipid phosphorus appears to a great extent to be governed by metabolic requirements of the body as a whole (Geschwind, Li, and Evans, 1950;

Campbell and Kosterlitz, 1949; Zilversmit and DiLuzio, 1952). Administration of vitamin B_{12} to hyperthyroid rats fails to alter the rate of phospholipid synthesis. Hence it must be concluded that the increased liver phospholipid synthesis obtained by feeding iodocasein is not directly attributable to a vitamin B_{12} deficiency.

The amounts of DNA and PNA per gm. of wet liver appears to be unchanged in the control, hyperthyroid and B_{12} supplemented rats (Table 2). In a recent report on the effects of vitamin B_{12} on nucleic acid metabolism in the rat, Rose and Schweigert (1952) observed that the DNA and PNA content per gm. of fresh liver were 20 and 10% lower than those of the control. However, these differences were not apparent when expressed per cell basis. In sharp contrast to these findings, Canzanelli, Rapport and Guild (1949) and Drabkin (1950) found a marked increase in the amounts of liver PNA per gm. of wet tissue on administering thyrotoxic materials to mature rats. A decrease in the liver PNA content, without alteration in the DNA level, follows a number of experimental conditions as fasting (Davidson, 1947), ischemia (Drochmans, 1947), administration of adrenocorticotropic hormone (Baker, Ingle, Li, and Evans, 1948) and poor protein diets (Mandel, Jacob and Mandel, 1950). Instead of a reduction in the absolute amounts of PNA, a cytoplasmic redistribution of this cellular substance can also occur as in the case of livers of rats on protein restriction (Muntwyler, Seifter and Harkness, 1950). The constancy of DNA in the present report is not unexpected, because more rigorous conditions as fasting (Davidson, 1947), protein poor diets (Mandel, Jacob and Mandel, 1949, 1950) fail to affect the level of this liver component. However, Muntwyler, Seifter and Harkness (1950) report an actual increase in the liver DNA content of rats on low protein diet.

Although the PNA content remains unaltered in the three groups (Table 2.), the rate of synthesis in the hyperthyroid group is increased nearly by 70% ($P < 0.01$) over the control. Supplementation with vitamin B_{12} (group III, Table 2.) lowers the relative activity of this fraction closer to normal values, probably indicating a sparing action by the vitamin for PNA turnover in the hyperthyroid rat. The ratio of the specific activity of PNA phosphorus to that of DNA in the control group is in excellent agreement with the findings of Brues *et al.* (1944). The concentration of P^{32} in the DNA fraction of all the three groups is in accordance with the observations of Davidson (1947), Brues, Tracy and Cohn (1944) and Hammarsten and Hevsey (1946) on the comparative inertness of this liver component.

Hyperthyroidism or supplementation with vitamin B_{12} do not alter either the concentration or activity of PP. The levels of P^{31} and P^{32} of this fraction are in close agreement with those reported by Singal *et al.* (1953).

This data confirm other reports that this fraction is metabolically the most active of the components in the acid-insoluble and nonlipid phosphorus (Spiegelman and Kamen, 1946, Friedkin and Lehninger, 1949).

DISCUSSION

The main effort of this investigation has been devoted to a study ofthe sequence of events that occur in the major liver phosphate fractions, six hours after the administration of the tracer to normal, hyperthyroid and B_{12} supplemented immature rats. Although it would be highly desirable to obtain information on the renewal or turnover times of the various phosphate fractions, it appears reasonable to assume that relative increases or decreases in the incorporation of P^{32} into the organic phosphates under conditions of the present report, reflect similar changes in the turnover rates.

In order to enlarge in detail on the changes observed in the P^{32} and P^{31} values of the total acid-soluble fraction, it would be necessary to isolate the major components of this fraction and evaluate their individual renewal rates. Nevertheless, on the basis of limited data available on this fraction, the evidence points to an inhibition of the uptake of P^{32} in the hyperthyroid animal and supplementation with B_{12} appears to off-set this impairment. Since no direct evidence could be obtained that a vitamin B_{12} deficiency is the primary cause of increased incorporation of P^{32} into the liver phospholipid, it should be of value to examine the relation between the turnover of tissue phospholipid phosphorus and fatty acid metabolism in the hyperthyroid rats. It must be made clear that our data do not reveal how hyperthyroidism accelerates the rate of incorporation of P^{32} into the liver PNA fraction. This may only be a part of the broader cytochemical alteration induced by administering thyrotoxic substances (Barker, 1951). The sparing action of vitamin B_{12} for PNA turnover observed in supplemented thyrotoxic rats is probably related to the findings of Koch-Weser and Popper (1949) and Stern *et al.* (1949) on the role of vitamin B_{12} in the genesis of pentosenucleic acid in the liver cells.

SUMMARY

Radioactive phosphorus as disodium hydrogen phosphate was administered to normal, hyperthyroid and B_{12} supplemented hyperthyroid immature rats. The animals were sacrificed six hours after the administration of tracer dose. The amount of phosphorus and radioactivity was determined in the total acid-soluble, phospholipid, DNA, PNA and PP fractions of the liver. Hyperthyroidism depressed the uptake of P^{32} in the total acid-soluble fraction compared to normal and supplementation with vitamin B_{12} tend to off-set this impairment. The rate of phospholipid syn-

thesis was increased in the thyrotoxic rats and was unaffected by supplementation with vitamin B_{12}. PNA synthesis was also increased in the iodocasein fed animals but the relative activity of this component fell close to normal values on supplementation with the vitamin. DNA and PP were unaffected by feeding thyroprotein and supplementation with B_{12}. An attempt has been made to interpret the significance of these changes.

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THE AGE OF BEGINNING REACTIVITY OF THE PITUITARY-ADRENOCORTICAL SYSTEM TO STRESS IN THE MOUSE[1]

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INTRODUCTION

IT IS well known that adult animals respond to stressing stimuli by the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary which in turn stimulates the adrenal cortex to produce and release glucocorticoids. The problem still remains as to when in the development of an organism the pituitary-adrenocortical system matures sufficiently to carry out this function. Although a functional inter-relationship between the hypophysis and the adrenal cortex has been shown in the fetus (Jost, 1948; Wells, 1948; Kitchell, 1950; Kitchell and Wells, 1952a, b; and others) there is abundant evidence that the homeostatic mechanism for bringing about activation of the adrenal cortex has not reached functional maturity at birth. Jailer (1949, 1950) reported that stress by cold gave no significant depletion of adrenal ascorbic acid in the infant rat until the sixteenth day of life, whereas he was able to secure such depletion with epinephrine on the eighth day and with ACTH on the fourth. Similar immaturity has been shown in the human infant (Reed, Venning and Ripstein, 1950; Jailer, *et al.*, 1951; Klein, 1951).

In the study of this problem various criteria for adrenocortical stimulation are available. It has been repeatedly demonstrated that a depletion of eosinophilic leucocytes follows the application of a stressing stimulus. The present study reports experiments designed to determine the age at which the pituitary-adrenocortical system in the infant mouse first becomes reactive to stressing by heat and epinephrine and to injections of ACTH, using depletion of circulating eosinophils as the criterion. A preliminary report of this work appeared earlier (Thompson and Blount, 1953).

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MATERIALS AND METHODS

Young white mice of a highly inbred local strain ranging in age from newly born to sixteen days were used. The technique for counting eosinophils was that of Speirs and Meyer (1949). This is a direct counting-chamber method that utilizes a special diluent fluid (Speirs, 1952). Individual experiments were limited to single litters because of the relative intra-litter uniformity of the eosinophil count (Speirs and Meyer, 1949). All experiments lasted only 4 hours following application of the stressing stimulus and the young were kept with their mother to allow them to nurse. The time of day at which the counts were taken was uniform in order to obviate the diurnal variation in eosinophil level (Halberg and Visscher, 1950; Speirs, 1953). Two sites were used for taking blood samples, the tail and the neck. In older animals, sufficient blood may be obtained for counting by severing the tail, and at all ages, sufficient blood can be obtained from the neck. Blood must be pipetted as quickly as possible from the flowing pool in order to avoid contamination with tissue fluids. The stimuli applied were three: heat, epinephrine and adrenocorticotrophic hormone (ACTH).

Animals were stressed by heat in an oven at between 38° and 40° C. Animals over three days of age were exposed for seven minutes, those less than three days for five minutes. The experiments with heat are divided into two categories. In the first and larger category, Series A, each litter studied was divided into control and test groups. Only one eosinophil count was performed on each individual, using blood obtained by severing neck vessels. This procedure was adopted because sufficient blood could not be obtained from the tail in younger animals. The second category, Series B, consisted of animals older than eight days in which a pre-stress and a 4-hour post-stress eosinophil determination was made on each animal.

Those animals receiving epinephrine were given 1.5 gamma/gm. of body weight. The dose of ACTH[3] was 0.5 USP units. In the epinephrine and ACTH studies only one count was performed on each animal, within litters divided into test and control groups.

Two methods were used in the calculation of results. In those experiments in which litters were divided into test and control groups, a percentage difference was obtained for each separate litter. In those studies in which both a pre-stress and a post-stress count were made on the same individual, the results are expressed as percentage change.

In considering the results obtained in this study, an interpretation of the data required an evaluation of the degree of change that is to be considered significant, and it is common practice to fix an arbitrary percentage level of depletion beyond which the stimulation of secretion of adrenocortical hormones is interpreted as being certain. In the present work a depletion of 40% is used, following the precedent of Jailer, Wong, and Engle (1951).

RESULTS

The experimental animals are divided, for the description of results, into three series: those stressed with heat, those stressed with epinephrine, and those injected with adrenocorticotrophic hormone (ACTH).

Stress by heat

The heat exposures used were just sublethal. The eosinophil counts of animals subjected to heat are in two categories; namely, Series A, those in

[3] The ACTH used was "Corticotrophin" of the National Drug Company.

TABLE 1. HEAT STRESS AND EOSINOPHIL COUNT AT DIFFERNT AGES

Age	Series A			Series B		
	Test and control groups One count per animal			Pre-stress and post-stress counts on same animal		
	Number of pairs	Percentage difference		Number of animals	Percentage change	
7 Days	33	+2	±3.8	—	—	
8 Days	54	+6	±4.1	—	—	
9 Days	35	+4	±7.5	46	+2	±4.6
10 Days	48	−12	±3.0	83	−35	±3.2
11 Days	44	−29	±2.0	34	−57*	±4.1
12 Days	30	−49*	±3.3	31	−58*	±3.7
13 Days	31	−54*	±2.4	35	−77*	±2.1
14 Days	30	−61*	±3.2	32	−81*	±3.0

* Reduction of 40% arbitrarily considered to indicate reaction to stress.
± Standard error.

which each litter was divided into test and control groups with only one count per animal taken; and Series B, those in which a pre-stress and a 4-hour post-stress count were obtained for each animal.

The results of studies on Series A are given in Table 1. In this series no significant fall in the eosinophil level following heat stress was noted in any litter less than ten days old. Individual litters in fact showed slight to moderate increases in the eosinophil count following heat that might be considered significant. Litters, aged 7, 8 and 9 days showed an average increase in the eosinophil count after stress of 2, 6 and 4% respectively. A change takes place about the 11th day. Later than the 10th day no litter showed an increase in eosinophils following treatment. On the 11th day some of the litters showed large decreases and the average, although not significant, was a reduction of 29%. On the 12th day the majority of litters showed a significant response to heat stress with an average drop of 49%. By the 13th day of life all litters showed a significant post-stress eosinopenia, and on the 14th day, the drop in the eosinophil count was of the magnitude expected for adult animals. The average percentage difference for 13 days was 54 and for 14 days was 61. Adult animals used for standardization of technique usually showed an eosinopenia of about minus 70%.

The results of experiments on Series B are given in Table 1. Simultaneous counts using blood from the tail and blood from the neck in individual animals in the absence of stressing stimuli show that there is no variation in the number of eosinophils in blood taken from the two sites. It may be seen that animals 9 days old had no post-stress fall in the eosinophil count (actually a rise of 2%) and 10-day-old animals showed a decrease of 35%. Significant decreases in the eosinophil counts of individual animals appeared

TABLE 2. ELSINOPHIL COUNTS FOLLOWING STRESS IN NEONATAL MICE
(Expressed as percentage differences from control)

Age	Heat stress	Epinephrine injection	ACTH injection
Less than one day	+2 ±1.3 (22)	−62* ±1.7 (21)	−85* ±1.4 (22)
One day	−13 ±2.1 (21)	−56* ±4.2 (20)	—
Two days	−21 ±4.1 (17)	−48* ±5.5 (19)	—
Three days	+11 ±1.4 (19)	−67* ±2.9 (20)	—

( ) Number of pairs of animals
± Standard error.
* Reduction of 40% arbitrarily considered to indicate a reaction to stress.

for the first time on the 10th day of life. Animals 11 days old showed an average decrease of 57%, which is significant. Animals 12 days old had a decrease in eosinophils of 58% those 13 days old of 77%, and 14-day-old animals had an eosinopenia of minus 81%.

The close agreement between the two groups of animals stressed with heat (Series A and B), demonstrates the validity of doing only one count per animal in Series A. On the basis of this, significant differences in the eosinophil counts of control and test groups which appear at about the 11th day may be ascribed to stress.

To aid in the evaluation of the series that follow, a group of neonatal animals were subjected to heat stress. Table 2 shows the percentage difference following stress in animals less than one day old to be plus 2%. For animals of 1, 2 and 3 days these differences were minus 13, minus 12, and plus 11% respectively.

Injection of epinephrine

Litters of mice, ranging in age from less than one day to three days, were subjected to the stress of epinephrine injection (1.5 gamma gm. of body weight). All litters were equally divided as control and test groups and only one count was done on each animal. All animals receiving epinephrine showed a fall in eosinophils in 4 hours; the vast majority of these decreases were significant. The average eosinopenia in animals less than one day old injected with epinephrine was minus 62%. The average percentage decrease in the eosinophil count for one-day-old animals was 56, for two-day-old animals 48 and for three-day-old mice 67 (Table 2).

Injection of adrenocorticotrophic hormone

The test animals of this group were injected with 0.5 USP units of ACTH. Only mice less than one day old were included in this series. The

litters were divided into control and test groups, and one count was performed on each animal. The average eosinopenia found 4 hours following the injection was minus 85% (Table 2).

In both the epinephrine and ACTH studies, control series of animals less than one day old injected with physiologic saline failed to show eosinophil depletions.

DISCUSSION

The present studies show that the pituitary-adrenocortical mechanism in the infant mouse is not activated by the stress of heat until the 11th day of life. The age at which heat first gives this activation has not previously been studied. Using cold on young rats Jailer (1949, 1950) was unable to find reactivity until the 16th day. He suggested that this failure may be correlated with immaturity of temperature regulation. It would seem that this would not in itself serve to explain the failure in the case of heat as the increase in body temperature would speed up the metabolic rate and this might be expected to increase the rate of consumption of adrenocortical hormones. According to the theory of Sayers and Sayers (1948: Sayers, 1950) this should result in activation of the pituitary.

The adrenal cortex is not the site of immaturity since it is capable of being stimulated on the first day of life by the injection of ACTH. Furthermore, it has been found that a significant depletion of eosinophils follows injection of epinephrine in mice less than one day old, which indicates that the pituitary is capable of adrenocorticotrophic hormone production and release. The production of a significant eosinopenia by epinephrine requires the participation of the anterior pituitary. It has been repeatedly shown by work on hypophysectomized animals that epinephrine is incapable of effecting release of glucocorticoids by direct action on the adrenal cortex (Recant, *et al.*, 1950; Cheng, *et al.*, 1949a; McDermott *et al.*, 1950a; 1950b).

It is clear that in the infant animal there is an immaturity in the mechanism for activation of the pituitary following heat stress but where this immaturity lies remains a question. Long (1947) has given evidence that in stress there is a stimulation of the autonomic nervous system and concomitant release of epinephrine, which is followed by activation of the anterior pituitary. The nature of the physiologic mechanism by which epinephrine might stimulate the anterior pituitary is uncertain. It is known that nervous connections are not necessary (Cheng *et al.*, 1949; Gersberg *et al.*, 1950; Fortier, 1951) and it is effective by direct application to pituitary tissue (McDermott *et al.*, 1950a, 1950b; Fortier, 1952). No single theory for the mode of stimulation of the pituitary by epinephrine is generally accepted. A review of one theory is given by Long (1952).

The great difference in the age at which reactivity to epinephrine begins

in mice (this report) as compared to rats (Jailer, 1950), is difficult to understand. A variation in degree of maturity of the two species at birth might account for a small discrepancy but not one of eight days. Jailer (1950) suggested that the level of circulating ACTH may be high just before and just after birth, which would inhibit the pituitary and prevent its activation. Another explanation might be that the pituitary in the newborn rat does not have sufficient stored ACTH that can be liberated in response to stimulation. This is supported by his failure (Jailer, 1951) to demonstrate detectable quantities of ACTH in the pituitary of the newborn rat. On the other hand the production of a significant eosinopenia in the newborn mouse with epinephrine demonstrates that neither of these explanations applies, at least in this species. The present study and the work of Jailer do agree in reporting a considerable lag between the age at which animals first react to epinephrine and the age at which they begin to react to temperature stress.

The chain of reactions between the reception of a heat stimulus and the discharge of adrenocorticoids is clearly incomplete in the newborn. This defect is not in the pituitary itself or in its control of the adrenal cortex as shown by the present experiments. This indicates that there is a pre-pituitary break in the chain between the reception of the stimulus and the discharge of ACTH by the pituitary. One possible defect may be an immaturity of the mechanism for bringing about the reflex secretion of epinephrine, which would in turn cause a delay in reactivity to stresses other than exogenously administered epinephrine.

SUMMARY AND CONCLUSIONS

Young mice from less than one to fourteen days of age were subjected to stressing by heat and epinephrine and to injections of ACTH. Eosinophil determinations were made before and four hours after stressing. Significant eosinopenia following heat did not occur until about the 11th day, and by the 14th day the adult pattern of eosinophil depletion was established.

Mice less than one day old showed significant depletion of eosinophils four hours after injections of epinephrine and of ACTH. The response to ACTH indicates a maturity of the adrenal cortex in the newborn mouse and the response to epinephrine is interpreted as indicating that at this age the pituitary is capable of ACTH production, storage, and release.

It is suggested that the failure of mice less than eleven days old to react to heat stimulation may be due to a pre-pituitary break in the chain between reception of the heat stimulus and the discharge of ACTH. A possible defect may be an immaturity in the mechanism for bringing about the reflex secretion of epinephrine following stress.

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A HISTOPHYSIOLOGICAL STUDY OF THE DEVELOPING ADRENAL CORTEX OF THE RAT DURING FETAL AND EARLY POSTNATAL STAGES[1]

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IN RECENT years, interest has turned to the question of the function of the endocrine glands in the fetus. That the fetal pituitary influences the size of the adrenal cortex is indicated by the fact that destruction or removal of the pituitary, in the last trimester of gestation, has resulted in smaller cortices than normal at the time of birth (Wells, 1948; Raynaud, 1950; Jost, 1953). Wells demonstrated that cortical size could be restored by administering adrenotropin to such fetuses. There is also indication that fetal adrenals secrete cortical steroids. For example, after unilateral adrenalectomy of the fetal rat, compensatory enlargement of the remaining adrenal occurred, and this hypertrophy could be prevented by the administration of cortisone (Kitchell and Wells, 1952a).

The present study was undertaken to determine at what time the first histophysiological evidence for hormone formation and secretion by the adrenal cortex of the fetal rat could be detected, and also whether there was any evidence for variation in secretory rate during fetal life and in the immediate postnatal period. A group of histochemical tests for lipids was used which has proved valuable in an analysis of the activity of the adult adrenal cortex under normal and experimental conditions (reviewed by Greep and Deane, 1949; Deane and Seligman, 1953). The observations on the histochemical preparations were supplemented by examination of routine histological sections.

To our knowledge, the only previous study utilizing more than routine fat stains on fetal adrenals was made by Nicander (1952). He made no chronological study, however, to determine when the special lipid reactions first appeared or whether they varied during development.

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MATERIAL AND METHODS

Animals. Adult female rats of the Sprague-Dawley strain were placed overnight with males, and those that showed evidence of having mated, by the presence of sperm in the vaginal smear, were isolated. The time of fertilization in those animals that became pregnant was assumed to be shortly after midnight (Everett, 1948). Two rats were killed on each day of pregnancy from $16\frac{1}{2}$ to $21\frac{1}{2}$ days after breeding (with the exception that only one was killed at $17\frac{1}{2}$ days), and the adrenals were taken from at least two fetuses, generally one male and one female. In addition, the adrenals were fixed from 5 newborn rats (2 males, 3 females) killed 2 to 6 hours after birth, 4 (2 males, 2 females) killed at $1\frac{1}{2}$ days, and 2 males killed at $2\frac{1}{2}$ days.

The fetal and newborn animals were killed by decapitation and, excepting at the $16\frac{1}{2}$ day stage, the adrenals were dissected free and fixed. At this one stage, the trunks of the fetuses were fixed.

Histological techniques.[3] From each animal, the left adrenal was fixed for 24 hours in Bouin's fluid, and the right was fixed for one week in 10% formalin buffered to neutrality. The glands fixed in Bouin's fluid were embedded in paraffin, sectioned serially at 10μ and stained with Harris' hematoxylin and eosin.

The formalin-fixed adrenals were sectioned at 25μ on the freezing microtome and washed thoroughly in several changes of distilled water. They were prepared in the following ways: (1) stained for 1 or 3 minutes with a 1% solution of sudan black B in 70% alcohol for total lipids; (2) stained by the Ashbel-Seligman (1949) procedure for carbonyl groups; (3) stained by the Schultz method for cholesterol; (4) mounted unstained for study under the fluorescence and birefringence microscopes to determine the presence of fluorescent substances and birefringent crystals in the lipid droplets. For procedures 1 and 4, control sections were extracted for $\frac{1}{2}$ hour at room temperature with acetone.

Adrenal volumes. In order to calculate the volumes of the whole adrenals and the cortices, drawings were made of adrenal sections, with the medullary areas outlined, by means of a camera lucida (110×) or of a projecting microscope (60×). Drawings were made of every 10th section. The areas of the whole section and of the medullary portions were determined with a planimeter. The area of cortical tissue in the section was then obtained by subtracting the medullary area from the total area. Estimates of whole adrenal, medullary and cortical volumes were then made by the following formulas:

$$\text{Actual area of section (cm.}^2\text{)} = \frac{\text{area of projected outline (cm.}^2\text{)}}{\text{linear magnification}^2}$$

$$\text{Volume (mm.}^3\text{)} = 10^3 \times \text{sum of areas (cm.}^2\text{)} \times \text{distance between sections (cm.)}$$

RESULTS

No significant differences, either histological or histochemical, were found between the adrenal glands of male and female rats during fetal and early postnatal life.

(a) *Histological observations.* The following description is based on a study of serial sections stained with hematoxylin and eosin. In rat fetuses of $16\frac{1}{2}$

[3] We wish to thank Mrs. Pauline R. Noble for valuable assistance with some of the histochemical procedures.

days, the adrenal glands consisted of encapsulated masses extending laterally from either side of the dorsal mesentery at the cephalic end of the kidney and at the level of the stomach and liver (Fig. 1). The parenchymal tissue consisted almost solely of rather large, faintly eosinophilic, epithelioid cortical cells. The nuclei were large and vesicular. Sinusoidal channels divided the cortical tissue into irregular cords; a few clusters of

FIG. 1. Cross section through 16½ day rat fetus; paraffin section, hematoxylin and eosin; ×80. The encapsulated adrenal gland (AG) lies lateral to the dorsal mesentery. A cluster of prospective medullary cells (arrow) may be seen outside of the gland to the left.

hematopoietic cells occurred in their walls. Medial to the adrenals in the mesenchyma, as well as occasionally within the glands, lay clusters of small basophilic cells—the prospective medullary tissue. The nuclei of these cells were smaller than those of the cortical cells and also more chromophilic (see Fig. 4).

Medulla. On successive days, the medullary nests migrated toward the

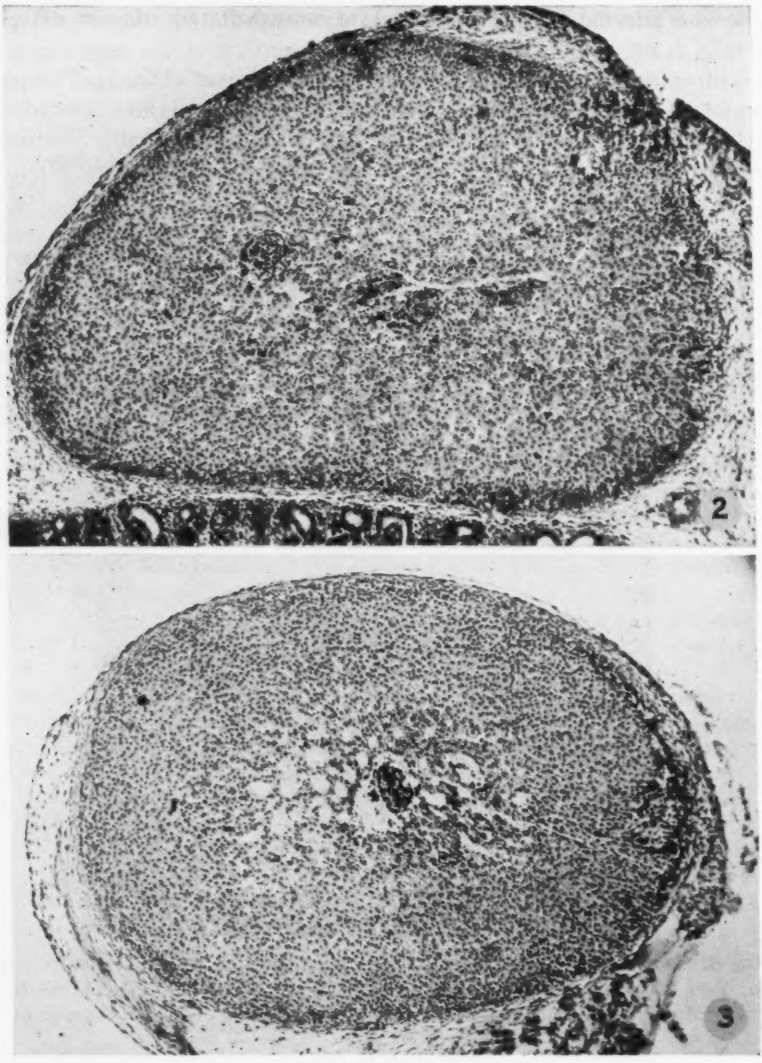

PLATE 1

Adrenal glands, paraffin sections, hematoxylin and eosin, ×80.

FIG. 2. Midsection of adrenal from a 20½ day fetus. The outermost cells (zona glomerulosa) are smaller than those of the fasciculata and reticularis. The medullary tissue occurs as scattered clusters of small cells near the center.

FIG. 3. Midsection of adrenal from a neonatal rat, ½ day old. The gland appears significantly smaller than that from the late fetal rat (compare Figs. 4 and 5). The dark central mass together with some of the pale areas consists of medullary tissue.

TABLE 1. AVERAGE VOLUMES OF SINGLE WHOLE ADRENALS, ADRENAL CORTICES AND MEDULLAS OF FETAL (F) AND NEONATAL (N) RATS, AS CALCULATED FROM MEASUREMENTS OF THE AREA OF EVERY 10TH SECTION OF SERIAL SECTIONS*

Age, days	No. rats	Whole adrenals, mm.³ ± S.E.†	Adrenal medulla, mm.³ ± S.E.	Adrenal cortex, mm.³ ± S.E.
F 16½	3	.080 ± .014	.002 ± .002	.078 ± .013
F 17½	2	.151 —	.002 —	.149 —
F 18½	4	.269 ± .026	.002 ± .000	.267 ± .025
F 19½	4	.357 ± .030	.002 ± .001	.355 ± .029
F 20½	7	.506 ± .035	.008 ± .001	.498 ± .035
F 21½	5	.571 ± .062	.012 ± .006	.559 ± .059
N ½	5	.436 ± .012	.019 ± .004	.417 ± .011
N 1½	4	.491 ± .030	.033 ± .005	.458 ± .026
N 2½	2	.449 —	.030 —	.419 —

* The volumes of adrenals of late fetuses (F 20½ and 21½) were compared statistically with those of neonatal rats (N ½, 1½ and 2½) (Snedecor, 1946). The differences as determined by the "t" test were significant, "t" = 2.85, d.f. = 21, P = <0.01. An analysis of variance was performed to test whether the differences in the total sample were attributable to age rather than to biological variability within the groups. It was found that 82% of the variation was attributable to age and was highly significant, F = 15.47, d.f. = 35, P = <0.01.

† Standard Error.

center of the glands and gradually coalesced (Fig. 2); yet, even at 2½ days after birth, the medullary tissue occurred in separate clusters surrounded by strands of cortical cells (Figs. 8 to 11). The medullary cells enlarged only slightly before birth, and the growth in volume of medullary tissue after 19½ days (recorded in Table 1) appeared to depend principally on cell division. Both hyperplasia and hypertrophy accounted for the further growth after birth.

Cortex. In the 16½ day fetuses, the cortical tissue consisted of two zones. A narrow layer immediately beneath the capsule consisted of relatively small, tightly-packed cells; this zone would correspond to the zona glomerulosa of the adult. Within a few days, the cells of the subcapsular layer enlarged and became organized into the small whorls characteristic of the adult. The rest of the cortical tissue was composed of somewhat larger cells organized in irregular cords. By 17½ days, this zone became subdivided into an outer fasciculata, in which the cords of cells were straight, and an inner reticularis, in which the cords were distorted (Fig. 4). The cells in the reticularis were indistinguishable from the cells of the fasciculata in staining properties or nuclear characteristics.

During fetal life the adrenal cortex enlarged steadily (Table 1). Growth of the cortex resulted from the steady hypertrophy of the cells, principally those in the fasciculata, as well as from cell division. Mitoses were most abundant during the first 3 fetal stages studied and were less common thereafter. Cell division occurred principally in the glomerulosa and in the fasciculata.

Immediately after birth there was a marked decrease in cortical volume,

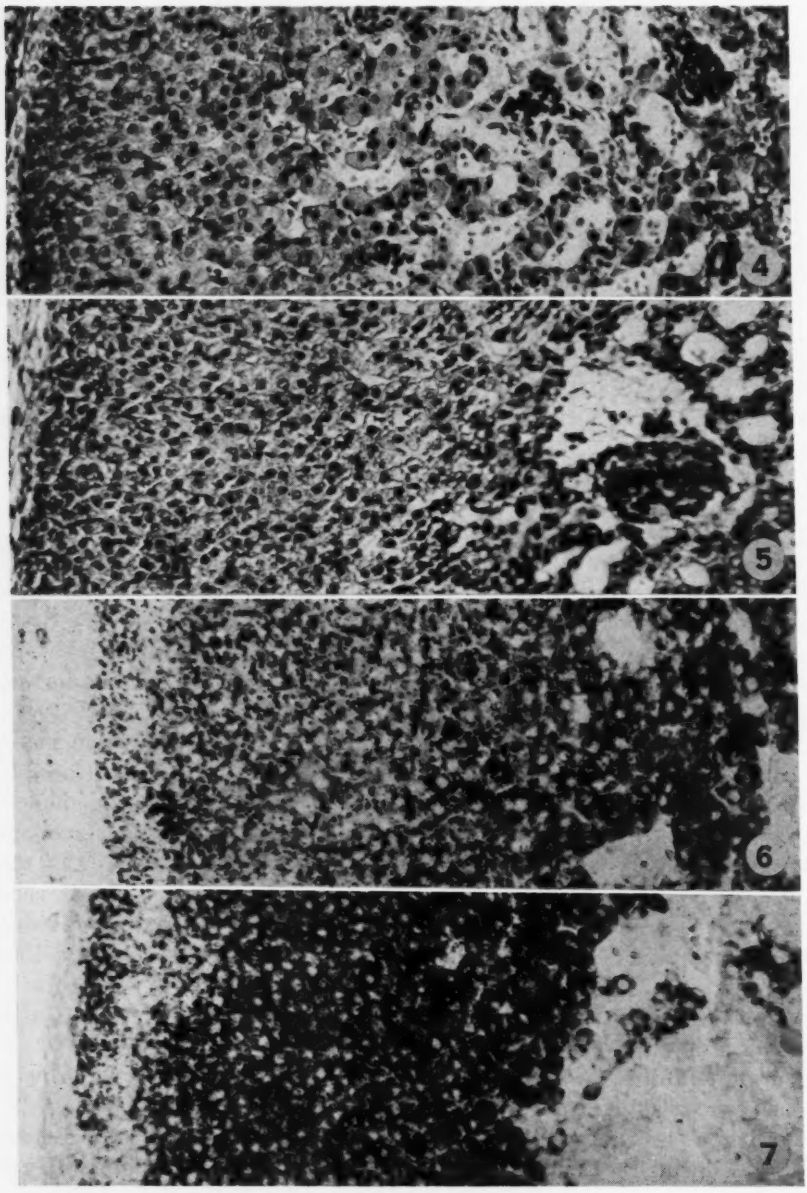

PLATE 2

FIGS. 4 and 5, paraffin sections, hematoxylin and eosin; Figs. 6 and 7, frozen sections, sudan black B; all ×215.

TABLE 2. EVALUATION OF THE AVERAGE INTENSITY OF THE LIPID REACTIONS IN THE ADRENAL CORTICAL CELLS OF FETAL (F) AND NEONATAL (N) RATS

Age, days	No. rats	Glomerulosa					Fasciculata					Reticularis				
		S	A-S	Sch	B	F	S	A-S	Sch	B	F	S	A-S	Sch	B	F
F 16½	2	(1)	—	—	—	—	(1)	—	—	—	—	(1)	—	—	—	—
F 17½	2	1	1	0	0	0	2	2	0	(1)	0	2	2	0	(1)	0
F 18½	4	1	1	0	(1)	0	2	2	(1)	1	0	2	2	0	1	0
F 19½	4	1	1	(1)	0	0	2	2	(1)	(1)	(1)	2	3	0	0	0
F 20½	8	2	1	1	(1)	(1)	2	2	(1)	(1)	(1)	3	3	(1)	(1)	(1)
F 21½	4	2	1	(1)	(1)	1	3	2	1	1	(1)	3	3	2	1	(1)
N ½	5	2	2	1	(1)	1	4	3	1	2	1	4	3	1	2	1
N 1½	4	3	2	1	(1)	1	4	3	2	3	2	4	3	2	3	2
N 2½	2	3	3	(1)	(1)	(1)	4	3	(1)	3	2	4	3	(1)	3	(1)

Key:

S = sudan black B
A-S = Ashbel-Seligman carbonyl reaction
Sch = Schultz cholesterol test
B = birefringent crystals } acetone soluble
F = fluorescent lipid droplets }

— no observation
0 no reaction
(1) weak reaction in scattered cells
1 weak reaction in all cells
2 moderate reaction in all cells
3 strong reaction in all cells
4 intense reaction in all cells

as shown in Table 1 (cf. Figs. 2 and 3). This decrease appeared to be the result of two factors: one, a conspicuous shrinkage of the cells of the fasciculata and reticularis; and, two, the disappearance of cortical cells lying among the medullary clusters (Fig. 5). Nevertheless, mitoses were still observed in the outer half of the cortex.

(b) *Histochemical observations.* A summary of our evaluation of the histochemical results on the cortices is presented in Table 2. These figures represent the averages of the independent evaluation by two of us. Although a trend is evident in these average figures, considerable variation in lipid reactivity existed between the adrenals in any one group of rats.

FIG. 4. Segment of adrenal gland from 18½ day rat fetus. The cells in the glomerulosa are small and tightly packed, whereas those in the fasciculata and reticularis are large. The cords in the fasciculata are straight, in the reticularis highly distorted.

FIG. 5. Segment of adrenal gland from a neonatal rat, ½ day old. The glomerulosa cells are somewhat enlarged in comparison to the fetal condition, while the fasciculata and reticularis cells are generally smaller, particularly in the inner portion.

FIG. 6. Segment of adrenal gland from 18½ day rat fetus. Small lipid droplets occur in all of the cortical cells, being most sparse in the transitional zone between the glomerulosa and fasciculata, and most abundant in the cells of the reticularis. The droplets also reacted with the Ashbel-Seligman reagent, and some in the fasciculata and reticularis exhibited a faint Schultz reaction and contained birefringent crystals. None was fluorescent.

FIG. 7. Segment of adrenal gland from a neonatal rat, ½ day old. Lipid is more abundant than in the fetal rat, both in the glomerulosa and inner zones. These droplets exhibited all of the special reactions (see Plate 3).

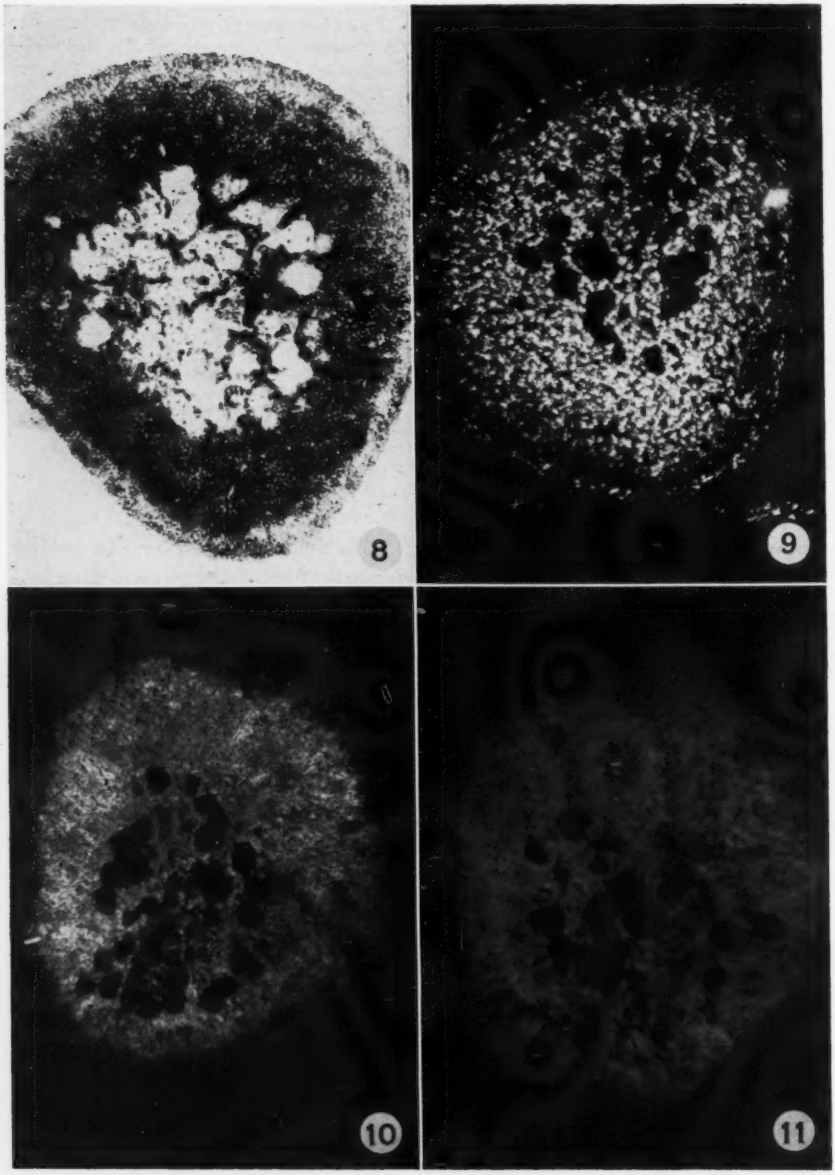

PLATE 3

Adrenal of a neonatal rat, 1½ days old; frozen sections prepared by several of the special methods for lipids; all ×80.

At 16½ days of fetal life, the adrenals appeared virtually fat-free, with only a few minute sudanophilic droplets occurring in some of the cortical cells. It appeared that they were somewhat more abundant in the outer rim of cells (glomerulosa) and in the innermost cells (reticularis) than in the intermediate fasciculata. Because of the paucity of lipid, none of the special histochemical tests was employed on sections of the 16½ day adrenals. By the next day slightly larger and more numerous droplets were present in the cells of the fasciculata and reticularis, although those in the glomerulosa remained small and sparse. The droplets stained a blue-purple by the Ashbel-Seligman carbonyl test, and occasional fine birefringent crystals occurred in the fasciculata. No Schultz reaction or fluorescence was detectable.

Thereafter until birth, there was a general increase in sudanophilic lipid in the cortex, with the glomerulosa continuing to stain more weakly than the fasciculata and reticularis (Fig. 6). By 18½ days, a lipid-free "transitional" zone became detectable between the glomerulosa and fasciculata. In some specimens the lipid droplets were larger and more numerous in the reticularis than in the fasciculata. After 18½ days, lipid droplets occurring in all regions of the cortex, in addition to displaying a carbonyl reaction and birefringence, gave the Schultz reaction; and after 19½ days some emitted a yellowish fluorescence.

After birth, the lipid content showed another striking increase (Fig. 7). As before birth, both the total lipids and the special reactions were more intense in the fasciculata and reticularis than in the glomerulosa (Figs. 8 to 11). The droplets appeared more abundant but remained small in the cells of the glomerulosa and fasciculata; those in the reticularis were as large as, or larger than, they had been before birth.

DISCUSSION

In the rat, the primordia of the adrenal cortices first appear on the dorsal mesentery on the 13th day of fetal life and grow rapidly thereafter (Pankratz, 1931). On the 17th day, the medullary cells begin to invade the cortical masses and then migrate in clusters toward the centers of the glands. In our preparations of 16½ day adrenals, the cortical tissue appears differentiated into a thin subcapsular layer of small cells and a central mass of larger parenchymal cells arranged in irregular cords. Within

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FIG. 8. Stained by the Ashbel-Seligman method for carbonyl groups.

FIG. 9. Unstained section photographed under the polarizing microscope.

FIG. 10. Unstained section photographed under the fluorescence microscope.

FIG. 11. Control for Fig. 10, unstained section extracted with acetone and photographed under the fluorescence microscope.

a few days, the cortex becomes organized into three zones; a narrow zona glomerulosa, a broad fascicular zone, and an equally broad reticularis. The cortical volume increases steadily until birth, whereupon it declines significantly (Table 1), as a result of shrinkage of the cells in both the fasciculata and reticularis and actual disappearance of many of the cortical cells between the medullary clusters.

Histochemically, at $16\frac{1}{2}$ days, only occasional small sudanophilic droplets occur in the cortical cells. The number of lipid droplets increases appreciably by $17\frac{1}{2}$ days and thereafter continues to increase slowly until birth. After birth there is a further marked increase. The distribution of the droplets in the three zones corresponds to the patttern observed in newborn rats (van Dorp and Deane, 1950). After the $17\frac{1}{2}$ day stage, many of the lipid droplets begin to display the carbonyl reaction of Ashbel and Seligman (1949). Within the next two days the Schultz cholesterol reaction, small birefringent crystals, and a yellowish fluorescence become detectable.

Although the chemical significance of most of the tests employed remains unsettled, the importance of the presence of these various reactions in the lipid droplets of fetal adrenals resides in the empirical evidence that they are somehow associated with hormone production (Deane, 1951; Deane and Seligman, 1953). It would therefore appear that significant production of hormones by the adrenal of the fetal rat is occurring at least by the $19\frac{1}{2}$ day stage, and perhaps even earlier. In a similar study on the developing adrenals of chick embryos, Dawson (1953) found that these special reactions had all made their appearance in some of the cortical cells by the 9th day of incubation (see also Case, 1952).

In addition, the cytological characteristics of the cortical cells in the fetal rats are compatible with the assumption of rapid release of hormones. That is, the cells are large, the lipid droplets are small, the carbonyl reaction is intense, and the cholesterol reactions (Schultz, birefringence) are relatively faint (Deane, 1951). This is particularly true of the fasciculata. Although the cells of the glomerulosa are somewhat smaller than those in the fasciculata, their droplets are likewise small and exhibit the histochemical characteristics associated with rapid secretion. After birth, the cells of the glomerulosa remain essentially unchanged, while those of the fasciculata shrink considerably. Nevertheless, the droplets remain small in the fasciculata as well as in the glomerulosa, whereas those in the reticularis tend to increase in size. These alterations may be interpreted to mean that the cells in the glomerulosa continue to secrete at the rate exhibited before birth, whereas those of the fasciculata secrete at a somewhat slower rate, and those of the reticularis decline still further in secretory activity.

The histophysiological indices of secretion by the cortical cells during fetal life are compatible with the evidence that the adrenal cortex produces some cortisone-like hormones in response to adrenotropin released by the fetal pituitary (Jost, 1953). Up to now, there has been no indication as to when such secretion begins. From a variety of experiments on adult rats, it may be assumed that such 11-oxygenated hormones are produced in the zona fasciculata (Greep and Deane, 1949).

In the adult rat, there is abundant evidence that the zona glomerulosa probably produces a hormone which influences electrolyte distribution; hormone secretion by the zone does not appear to be under significant control by the anterior pituitary (Deane, 1951; Eichner, 1953). The glomerulosa of the fetal rat is distinguishable from the fasciculata both morphologically and histochemically. Whether or not these differences indicate differences in product or in control remains to be determined. The only relevant observation is that of Kitchell and Wells (1952b), who found that the injection of fetuses with an aqueous solution of adrenotropin caused cell enlargement in the glomerulosa as well as in the fasciculata. At least two interpretations of this result appear possible: (1) the glomerulosa of the fetus, unlike that of the adult, may be controlled significantly by adrenotropin (although it may secrete a hormone differing from that of the fasciculata); or (2) the injected fluid may have disturbed the electrolyte balance in the fetuses.

The reticularis may be interpreted as possesssing less secretory activity than the outer portions of the cortex, by virtue of the frequent occurrence of large lipid droplets in its cells and its postnatal atrophy. Otherwise, the cells of the reticularis are not distinguishable from those in the fasciculata. None of our observations confirms the suggestion of Howard (1938) and Benua and Howard (1950) that the innermost zone of the adrenal cortex should receive the special designation of "juvenile cortex." Howard has suggested that this inner zone may secrete an androgen. Kitchell and Wells (1952b), however, have demonstrated that adrenalectomy of male fetuses failed to affect the growth of the seminal vesicles, although castration resulted in diminished growth.

Despite these precautionary remarks about attributing any special function to the innermost region of the fetal cortex, the fact remains that, while cells of the fasciculata shrink somewhat after birth, the cells of the reticularis not only atrophy but also gradually disappear (see also van Dorp and Deane). Absolute shrinkage of the adrenal cortex also occurs in the human after birth, and this involution is principally a result of atrophy and disappearance of the innermost zone, or "fetal cortex" (Swinyard, 1943). The most likely explanation of this cortical involution seems to us

to be a decline in pituitary activity after birth. Another possibility, less probable on the basis of current information, is that the decline reflects the removal of maternal adrenotropin, small quantities of which may cross the placenta to summate with the hormone emanating from the fetal pituitary (Jones, Lloyd and Wyatt, 1953).

SUMMARY AND CONCLUSIONS

Adrenal glands were obtained daily from fetal and neonatal rats from $16\frac{1}{2}$ days of gestation until $2\frac{1}{2}$ days after birth. Histological study shows that the glands grow steadily from $16\frac{1}{2}$ days to birth, principally as a result of enlargement of the cortical mass; after birth there is a significant decline in the size of the cortex. By $18\frac{1}{2}$ days, 3 zones may be distinguished in the cortex: a narrow zona glomerulosa, a broad fasciculata, and an equally broad reticularis.

Sudanophilic lipid droplets occcur in the fetal cortical cells at $16\frac{1}{2}$ days of gestation, and their number increases throughout the interval studied. Lipid is sparser in the glomerulosa than in the fasciculata and reticularis. Between $17\frac{1}{2}$ and $19\frac{1}{2}$ days of gestation, the Ashbel-Seligman carbonyl reaction, the Schultz cholesterol reaction, birefringent crystals and a yellowish fluorescence begin to appear in the lipid droplets. The histochemical and morphological evidence indicates that active secretion by the cortical cells occurs at least by $19\frac{1}{2}$ days of gestation, and further, that activity diminishes immediately after birth.

The possibility of separate functions for the glomerulosa, fasciculata and reticularis is discussed.

Addendum

Since this manuscript went to press, a report by Knobil and Briggs (1954) has called into question the hypothesis that the only stimulus to the fetal adrenal cortex comes from the fetal pituitary, since they obtained evidence that maternal adrenotropin crosses the placental barrier. For our observations, the principal significance of their results is that the decline in cortical volume after birth most likely reflects the removal of maternal adrenotropin.

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CYTOLOGICAL EFFECTS OF TESTOSTERONE PROPIONATE ON EPITHELIUM OF RAT SEMINAL VESICLES

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INTRODUCTION

MOORE, Hughes, and Gallagher (1930) made a comprehensive study of the effect of castration and male hormone replacement on the seminal vesicles of the rat. These investigators observed nuclear changes as well as reduction in cell height in the secretory epithelium following gonadectomy. Furthermore, the injection of testis extract brought about the rebuilding of degenerate vesicles to their functional condition. The epithelial cell height of seminal vesicle tissue is closely correlated with the level of male hormone present in body fluids.

In this study observations were made on the action of male sex hormone on nuclear diameter, nucleolar changes, cell height, and mitotic activity of the secretory epithelium of seminal vesicles of sexually mature rats. Intact, castrate and hormone-treated castrate animals were used to obtain the cytometric data presented here.

MATERIALS AND METHODS

A total of 114 male rats of the Holtzman-Rolfsmeyer strain was used in this investigation. At the time of gonadectomy, the animals weighed 250–275 gm. The rats were maintained on Purina Laboratory Chow and raw carrots. The following fixatives, staining techniques, and cytometrical methods were used:

Nuclear Measurements and Cell Height. Average diameters of equivalent circular cross sections of nuclei were calculated from nuclear areas obtained by camera lucida drawings on coordinate paper ruled in square millimeters. In as much as the sections were cut in various planes with respect to the ellipsoidal epithelial nuclei, the average equivalent circular nuclear diameters were therefore less than the maximal values. From each animal the average nuclear diameter was obtained from 200 nuclei by making measurements of 50 cells located in 2 sections on each of 4 slides. Cell heights were measured with an ocular micrometer from the same histological preparations used for nuclear di-

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ameters and the average value per individual was obtained by measuring 60 cells located in 6 sections on each of 3 slides. For these measurements, pieces of one lobe of each seminal vesicle were fixed in Allen's modification of Bouin's fluid (Painter, 1922). Sections were cut at 10 μ and stained with Heidenhain's iron hematoxylin. Eighty-four animals were used for this part of the investigation. Rats in groups of 4 were killed at 0, 6, 12, 24, 36, 48, and 60 hours as well as 5, 10, 20, 30, and 38 days after castration. In addition, observations were made on one animal which was gonadectomized at 25 days of age (55 gm.) and killed 172 days later. Data were also obtained from hormone-treated animals which had been castrated from 38 to 51 days prior to hormone therapy. Five hundred μg. of testosterone propionate in oil[2] (T.P.) was injected subcutaneously daily. These animals were killed at intervals of 6, 12, 24, 36, 48 and 60 hours as well as 5, 10, and 20 days after initial hormone administration. There were 3 animals in the 10-day group in this series and 4 in all others.

Mitotic Activity. Twenty-seven rats weighing approximately 275 gm. at time of castration were used for the observations on mitotic activity. The seminal vesicles were allowed to atrophy for 38 days before hormone treatment (500 μg. per day). Animals in groups of 3 were killed at 0, 24, 36, 48, and 60 hours and 5, 10, and 20 days after the initial injection. In addition, 3 rats received daily 1000, 2000, and 4000 μg. respectively of T.P. for 48 hours. Data were also obtained from 3 sexually mature intact males. Animals were injected subcutaneously with 0.1 mg. colchicine per 100 gm. body weight 6 hours prior to killing, according to Burkhart (1942). Pieces from each gland were fixed in Bouin's picro-formol, sectioned at 10 μ and stained with Delafield's hematoxylin and eosin Y. The percentage of mitotically dividing nuclei was obtained by random selection of 1000 cells per animal. In those cases in which there were more than 1% dividing cells, the tissues were sectioned at 4 μ and a total of 5000–7000 cells were counted per rat.

Nucleolar Activity. Nucleolar activity in the secretory epithelium of seminal vesicle tissue was evaluated in the previously described intact, castrate, and hormone-treated animals used in furnishing data on nuclear diameters and cell heights. Material was prepared in 3 fixatives: (1) Allen's modification of Bouin's fluid followed by Heidenhain's iron hematoxylin stain gave adequate preparations for nucleolar studies of the secretory epithelium of rat seminal vesicles (Painter, 1922). One of the problems encountered during this investigation was the differentiation of the nucleolus from chromatin aggregations in pyknotic nuclei of castrates. Extensive destaining was found to be an aid in nucleolar identification, especially in the late stages after gonadectomy. In many cases the nucleoli retained the hematoxylin longer than did the chromatin and thus were more clearly seen. (2) End-point staining with thionin was used at pH values ranging from 1.0–4.4 following Carnoy's fixative as suggested by Davenport, Windle and Rhines (1947). The best preparations with this method for the study of nucleoli in castrates were obtained at pH 3.6, 4.0 and 4.4. (3) Feulgen's technique as modified by Lhotka and Davenport (1947) was useful in identifying the nucleolus in some cases because of the ring of desoxyribonucleic acid surrounding this structure. Observations were also made on sections which had been incubated for 6 hours at 30° C. in McIlvaine's buffer (pH 6.5) containing 0.1 mg. of desoxyribonuclease per ml. (Barton, 1952). In addition, tissues were deparaffinized, hydrated, and placed in 0.2 N HCl for 1 hour at 58° C. This was followed by washing and overnight treatment at 30° C. in buffer (pH 6.5) containing 0.2 mg. desoxyribonuclease per ml. Control slides were placed in HCl solution (Barton, 1952). Tissues pretreated with the enzyme or acid were stained with iron hematoxylin

[2] Peranderen, Ciba Pharmaceutical Products, Inc., Summit, N. J.

or 0.5% toluidine blue in 5% ethyl alcohol. The results obtained indicate that not all of the Feulgen positive material was removed by desoxyribonuclease under the conditions used.

RESULTS

The results of this investigation, with the exception of the observations on nucleoli, are summarized in Table 1 and Figure 1. Equivalent nuclear diameters and cell heights of the secretory epithelium are presented as means with their standard errors. The data for mitotic activity presented

TABLE 1. NUCLEAR DIAMETER, CELL HEIGHT AND MITOTIC ACTIVITY OF SECRETORY EPITHELIUM OF RAT SEMINAL VESICLES

Effect of castration			Effect of testosterone propionate			
Time after castration	Nuclear diameter mean ±S.E.* (μ)	Cell height mean ±S.E.* (μ)	Time after initial treatment	Nuclear diameter mean ±S.E.* (μ)	Cell height mean ±S.E.* (μ)	Mitotic activity %
0 hours	4.8±0.1	19±0.5	0 hours	3.8±0.2	8±0.4	0
6	5.1±0.0	17±0.4	6	4.1±0.1	8±0.3	—
12	5.0±0.2	17±0.4	12	4.5±0.1	9±0.3	—
24	4.7±0.1	15±0.4	24	4.9±0.1	10±0.4	0
36	4.6±0.0	12±0.0	36	5.3±0.1	13±0.4	0
48	4.6±0.1	12±0.6	48	5.7±0.2	14±0.4	3.8
60	4.3±0.3	11±0.5	60	5.8±0.1	17±0.3	7.5
5 days	4.4±0.1	9±0.0	5 days	5.6±0.1	17±0.4	1.4
10	4.3±0.1	9±0.0	10	5.3±0.1	17±0.4	0.3
20	4.4±0.1	9±0.5	20	5.4±0.1	18±0.8	0.1
30	4.0±0.2	8±0.4				
38	3.8±0.2	8±0.4				

* S.E. = Standard Error.

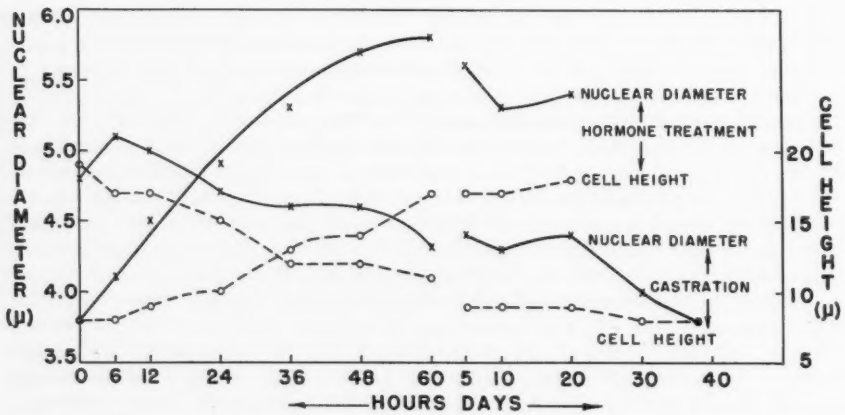

FIG. 1. Effects of castration and male sex hormone on secretory epithelium of the seminal vesicle.

in Table 1 represent average values. It was noted that there was a high degree of variability in the number of dividing cells in various areas of the same organ under androgen stimulation. It is possible that this may be related to differences in cell permeability and vascular response in different regions of the seminal vesicles to male sex hormone. The "t" test was used in the statistical analysis of the data presented and values of "t" were taken from Snedecor (1946).

The ellipsoidal nucleus of seminal vesicles of intact rats usually contained 1 or 2 heavily staining nucleoli (Fig. 2). These structures were variable in shape, but generally were quite large and distinct from chromatin granules. At this stage the nucleolus was discerned with ease in almost every cell.

Six as well as 12 hours after castration it was not possible to observe any changes in the nucleoli of the seminal vesicle epithelium. These structures were comparable to those seen in intact animals and were visible in almost every cell studied. However, 24 hours following gonadectomy a slight reduction in size of an occasional nucleolus was seen. Nevertheless, most of the nuclei contained large nucleoli and clumping of the chromatin material had not begun. Twelve hours later the nucleolar conditions of the previous stage persisted, and the first indications of chromatin flecking were observed in a few nuclei. In the 48 hour castrates many of the nucleoli were somewhat reduced in size as compared to those in intact animals, but other nuclei retained these structures comparable to those of the intact animals. At 60 hours after castration most of the nucleoli were small and some nuclei were flecked or pyknotic. Five and 10 days following gonadectomy, the majority of nucleoli were reduced in size and rather extensive clumping of nuclear chromatin had taken place. At 20 (Fig. 3) and 30 days after castration the nucleoli were in about the same condition as in the 5- and 10-day castrates, but the nucleus was somewhat more pyknotic. This same condition was present in the 38-day castrates except that the clumping and general pyknosis of the nuclei were more apparent.

No visible changes in the nucleolus or chromatin distribution were noted 6 hours after T.P. treatment of 38-day castrates. However, 12 and 24 hours following hormone injection, some of the nucleoli had increased slightly in size, but many were unchanged. The chromatin of a great number of nuclei was still flecked during this period in the process of cellular reactivation. Thirty-six hours after the initial treatment of these castrates, most of the nucleoli were of considerable size and in some cases were similar to those of intact animals. Nevertheless, at this stage the chromatin of many nuclei was often in aggregations. Twelve hours later all the nucleoli were large and the nuclei had lost their flecked appearance. From a histological viewpoint, they were similar to those in cells of intact rats. Following this,

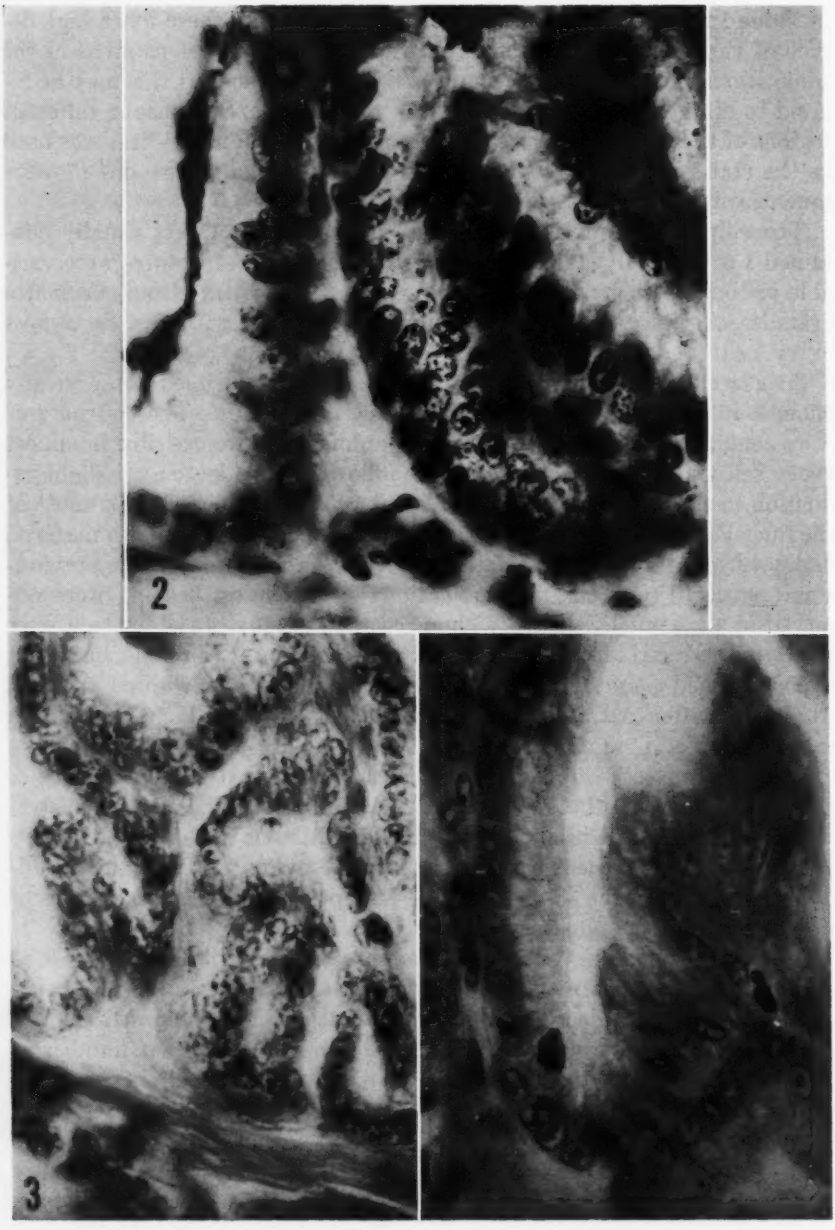

PLATE I

no further nucleolar alterations were noted in the epithelium after 2.5-, 5-, 10-, and 20-days of testosterone propionate treatment (Fig. 4).

DISCUSSION

As shown in Table 1 and Figure 1, 6 hours after castration the nuclear diameter showed an increase of 0.3 μ ($P=0.05$) and the cell height a decrease of 2 μ ($P=0.01$), whereas 18 hours later the former had decreased 0.1 μ (not Sig.) and the latter 4 μ ($P=0.01$). These morphological changes are related to the cessation of the secretory function of the gland as shown in earlier investigations by Porter and Melampy (1952) and Melampy and Cavazos (1953). However, the nuclear diameter decreased 0.5 μ ($P=0.10$) in the 60 hours following gonadectomy which represents a 10% change when compared to the average nucleus in the secretory epithelium of intact animals. Likewise the cell height showed a 42% decrease ($P=0.01$) at this time. In the 35.5 day interval between 60 hours following castration and the end of the experiment a further drop of 12% ($P=0.10$) was observed in the nuclear diameter and 27% in cell height ($P=0.01$). The total nuclear alteration of 1 μ ($P=0.01$) during 38 days represented a 21% change whereas the 11 μ ($P=0.01$) reduction in height was 58%. The average nuclear diameter was $4.1\mu \pm 0.2$ in the case of an immature animal castrated at 25 days of age (55 gm.) and examined 172 days later.

In the case of the hormone-treated animals the nuclear diameter increased 18% ($P=0.01$) in the 12 hours following administration of the hormone and the cell height 13% ($P=0.10$). The epithelium showed in the 24 hours after the initial injection an increase of 1.1 μ ($P=0.01$) in nuclear diameter and 2.0 μ ($P=0.01$) in cell height. By 60 hours the diameter had increased 53% ($P=0.01$) and the cell height 113% ($P=0.01$) above the values of the 38-day castrate animal (Table 1). After 60 hours the nuclear diameter showed a decrease whereas the cell height remained relatively constant at a value comparable to that of the intact animal (Fig. 1). The nuclear diameter of the hormone-treated castrate reached the value of the intact animal approximately 24 hours after the initial injection of the hormone and remained somewhat larger for the duration of the experiment.

Explanation of Plate I

Photomicrographs of rat seminal vesicle tissue fixed in Allen's modification of Bouin's fluid and stained with Heidenhain's iron hematoxylin. ×870.

Fig. 2. Section from an intact animal showing large nuclei with prominent nucleoli.

Fig. 3. Effects of 20-days castration on the nuclei and nucleoli of the secretory epithelium.

Fig. 4. Reactivation of the secretory epithelium of a 38-day castrate which had received testosterone propionate for 20 days.

As shown in Table 1, the increase in nuclear diameter following hormone injection is related to the nuclear activity associated with the mitotic cycle as well as preparation of the interphase nucleus for its role in the metabolic processes of the cell. A maximal mitotic activity of 7.5% occurred 60 hours after initial hormone treatment under the conditions of these experiments (Table 1) and the greatest nuclear diameter of 5.8 μ was noted at this time. The average nuclear diameter showed a decrease of 0.4 μ ($P = 0.01$) between the interval following 60 hours and 20 days of hormone treatment (Fig. 1). This change may be interpreted in terms of the fall in mitotic activity as well as the physiologic adjustment of the secretory epithelium to a constant amount of male hormone during this period. In the case of rats receiving 1000 and 2000 μg. of T.P. daily for 48 hours the mitotic activity was 4.0% and 3.7% respectively. However, a value of 11.6% was found in the animal receiving 4000 μg. daily during the same interval.

The cytological changes following hormone treatment summarized in Table 1 and Figure 1 are related to the functional reorganization of the cell following the relatively inactive castrate condition as well as maintenance of the secretory activity of the seminal vesicles. The results of this study strongly suggest a direct relationship between the nucleolus and the functioning of the secretory epithelium as indicated by the presence of 1 or 2 large, heavily staining nucleoli in each nucleus of both intact and hormone-treated animals (Figs. 2 and 4) and the reduction in size of these structures in the pyknotic nuclei of the non-secreting epithelium of the castrate (Fig. 3). The findings presented here are of interest in terms of the suggestions made by Caspersson (1950) and Brachet (1952) relative to the importance of the nucleus to protein synthesis.

The effects of testosterone propionate on the nuclear enlargment of the secretory epithelium of the seminal vesicles is similar in this respect to the action of other steroid hormones. For example, Hooker and Forbes (1947) have shown in the endometrium of the castrate mouse that progesterone transforms fusiform, dense, stromal nuclei with clumped chromatin and no visible nucleoli into plump, oval nuclei with conspicuous nucleoli and fine, evenly dispersed chromatin particles. Salvatore (1950) in an investigation of uterine gland cells of the rat found that estrone induced nuclear hypertrophy. According to Bullough (1952) androgens are among the substances which stimulate the rate of cell division by direct mitogenic action on cells and indirectly through the mobilization of carbohydrate. It is of interest that Rudolph and Samuels (1949) have shown that T.P. in oil causes an increase in the fructose content of seminal vesicle tissue 10 hours after hormone administration. Changes in permeability and vascularization of the seminal vesicles following androgen administration probably

contribute to increased availability of metabolites which may be required for mitotic activity. According to Roberts and Szego (1953) the most prominent early changes which occur in the target organ under the influence of the steroid hormones appear to be alterations in permeability and in carbohydrate metabolism.

SUMMARY

A cytometric study was made of the effects of castration and hormone replacement on the secretory epithelium of seminal vesicles of sexually mature rats. Average diameters of equivalent circular cross sections of nuclei were calculated from nuclear areas obtained by camera lucida drawings. Cell heights were measured with an ocular micrometer. Twenty-four hours following gonadectomy the average nuclear diameter showed an insignificant decrease of 0.1 μ whereas cell height was reduced by 4 μ ($P=0.01$). The diameter fell 0.5 μ ($P=0.10$) and the height 8μ ($P=0.01$) in 60 hours after castration. A total reduction in nuclear diameter of 1 μ ($P=0.01$), and in cell height of 11 μ ($P=0.01$), was noted during the 38-day period of observation.

In the case of hormone-treated animals (500 μg. testosterone propionate in oil daily) the nuclear diameter increased 0.7 μ ($P=0.01$) and cell height 1 μ ($P=0.10$) in the 12 hours following the initial administration of the hormone. By 60 hours the diameter had increased 2 μ ($P=0.01$) and the height 9μ ($P=0.01$) above the value of the 38-day castrate. After this time the nuclear diameter showed a decrease whereas the cell height remained relatively constant at a value comparable to that of the intact animal.

Sixty hours after gonadectomy most of the nucleoli were small and some nuclei were pyknotic. In the 38-day castrate general pyknosis was apparent. Forty-eight hours after initial hormone treatment the nucleoli were large and the nuclei had lost their pyknotic characteristics.

No mitoses were observed in the secretory epithelium of castrates, or the 24- and 36-hour hormone-treated animals. A mitotic activity of 7.5% was noted after 60 hours. Following this maximum there was a sharp decline in mitoses and in the case of animals treated with male hormone for 20 days a value of 0.1% was observed.

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EFFECTS OF VARIOUS HORMONES ON THE AMOUNTS OF PHOSPHORUS CONTAINING FRACTIONS OF THE BRAIN (PHOSPHOLIPID, NUCLEOPROTEIN, TOTAL ACID-SOLUBLE PHOSPHORUS COMPOUNDS)[1]

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THE purpose of the investigation here described was to ascertain whether the phospholipid content of the brain is a fixed amount, or an amount subject to changes of the hormone content of the brain tissue. The method used did not reveal any changes of the phospholipid content of the brain after administration of several hormones. It was, however, observed that the phospholipid content of the brain increases after a single intraperitoneal injection of corticotropin (ACTH) and decreases afterpr olonged administration of the thyrotropic hormone of the pituitary gland (TSH).

METHOD

The contents of phospholipid, nucleoprotein, and total acid-soluble phosphorus containing compounds of the brain were ascertained following a method described by King (1946) with minor modifications. The phospholipid fraction contained lecithins, cephalins, and sphyngomyelins. The nucleoprotein fraction contained ribo- and desoxyribonucleic acid and a small amount of phospho protein (Schmidt *et al.*, 1945).

The total acid-soluble phosphorus compound fraction contained nucleotides, inorganic phosphate, and phosphate esters.

Experimental Animals

Three to 4 months old male and female albino mice belonging to CF 1 strain of Caworth Farm were used. All animals received a mixed diet consisting of dog pellets (*G.L.F. Cooperative*), milk, bread, and vegetables *ad libitum*.

Fractionation of the Brain

The animals were decapitated. The surface of the forebrain was rapidly dried on a blotter and an aliquot of about 250 mg. was either dropped into 5 ml. of trichloracetic acid solution containing 10 grams of acid per 100 ml. of distilled water, or was dropped into 2 ml. of Bloor's solution (Brante, 1949). This solution contained 75% ethyl alcohol and 25% ethyl ether.

The suspensions were rapidly homogenized by means of a glass apparatus of Potter.

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Extraction of the total acid-soluble phosphorus compounds.—The homogenate containing trichloracetic acid was repeatedly shaken in the refrigerator for 15 minutes. After centrifugation at 3,000 r.p.m. the residue was resuspended twice in 5 ml. of a trichloracetic acid solution containing 5 mg. of acid per 100 ml. of distilled water for 10 minutes. Thereafter, the residue was twice resuspended in 5 ml. of distilled water for ten minutes. The supernatant fluids were pooled and diluted to 25 ml. with distilled water.

Extraction of the phospholipid fraction.—The residue and the homogenate containing only Bloor's solution were extracted by a micro-reflux apparatus with 10 ml. of Bloor's solution for 30 minutes. Extraction was repeated twice for 15 minutes using 10 ml. of fresh solvent. The supernatant fluids were pooled and brought to a volume of 30 ml. with the solvent. This extraction was sufficient to remove at least 99% of the phospholipid content of the brain (Brante, 1949).

Significant differences in the phospholipid content of the fractions were not detected between the fresh samples and the samples obtained after the extraction of the total acid-soluble phosphorus compounds.

Extraction of the nucleoprotein fraction.—The residue obtained after the extraction of the other two phosphorus containing fractions was brought into solution by oxidation with 2 ml. of a 70% perchloric acid solution (King, 1946).

Total phosphorus content of the brain.—Fifty mg. aliquots of the forebrain were dropped into 2 ml. of a 70% perchloric acid and dissolved by heating. Occasionally this procedure was preceded by a digestion in concentrated sodium hydroxide solution and by neutralization.

Phosphorus estimation.—Two ml. of a 70% perchloric acid solution (King, 1946) were added to two ml. samples (or the solid material) of the four fractions. After complete oxidation (King, 1946) the phosphorus content of the samples was estimated following the method of Fiske and Subbarow (1929) as described by King (1946).

Corrections

The blood remaining in the brain after decapitation and draining was estimated by the method of Ashby and Chen (1943). The amounts of the various fractions in the blood contained in the brain were sufficiently small not to interfere significantly with the phosphorus content of the brain fractions. The maximum interference was observed in the total acid-soluble phosphorus compounds, but even here it did not exceed 4%.

Administered Substances

The following substances were given either by intraperitoneal or intramuscular injections. The amounts were given in mg. per 50 gram body weight. With the exception of ACTH prolonged administration of the substances was chosen.

Corticotropin (ACTH, Acthar; Armour and Co.) a) ACTH was given in a single intraperitoneal injection in amounts of 1 mg. 15 minutes before decapitation; and b) ACTH was given intramuscularly in amounts of 1 mg. three times a day for 4 to 14 days.

TSH (thyrotropic factor, Armour) was given in amounts of 1 mg. once a day for 7 to 21 days.

Growth Hormone (Armour) was given in amounts of 1 mg. once a day for three weeks.

Pitocin (Parke, Davis and Co.) was given in amounts of 0.4 International Unit once a day for 10 days.

Pitressin (Parke, Davis and Co.) was given in amounts of 0.4 International Unit once a day for 10 days.

Follicular Stimulating Hormone of the pituitary gland (Parke, Davis and Co.) was given in a single injection in amounts of 0.4 mg. for 10 days.

Desoxycorticosterone Acetate (Roche-Organon) was given in amounts of 0.1 to 0.4 mg. once a day for 5 and 10 days.

Testosterone Propionate (Schering) was given in amounts of 0.1 to 1 mg. once a day for 5 and 10 days.

Progesterone (Schering) was given in amounts of 0.1 to 0.4 mg. once a day for 5 and 10 days.

Estradiol Benzoate (Schering) was given in amounts of 0.1 to 0.4 mg. once a day for 5 to 10 days.

21-Acetoxy-Pregnenolone (Schering) was given in amounts of 0.1 to 0.4 mg. once a day for 5 and 10 days.

11-Dehydro-17-Hydroxycorticosterone-21-Acetate (Cortone, Merck) was given in amounts of 0.4 and 1 mg. three times a day for 5 to 10 days.

17-Hydroxy-Corticosterone-21-Acetate (Merck) was given in amounts of 0.4 and 1 mg. three times a day for 5 and 10 days.

Adenosinetriphosphate (ATP, sodium salt) was given in amounts of 1 mg. three times a day for 5 and 10 days.

In each group of experiments ten or more mice were used.

RESULTS

Controls.—The average phosphorus content of seventy control brains was 447 mg. phosphorus per 100 grams brain (wet weight). The observed amounts approximated the calculated sum of the phosphorus contents of the three fractions. The percentage distribution of the phosphorus in the three fractions was: 60% (268 mg.) in the phospholipid fraction; 23.5%

TABLE 1. EFFECTS OF VARIOUS SUBSTANCES ON THE PHOSPHORUS CONTAINING FRACTIONS OF THE BRAIN

Substance injected	Phosphorus content of the various fractions expressed in per cent of control[1]		
	Phospholipid	Nucleoprotein	Total acid-soluble phosphorus containing compounds
Various[2]	96 to 105	95 to 105	96 to 104
ACTH prolonged administr.	97	98	97
Single injection (15 min.)	126	102	99
TSH	86	90	92

[1] The standard error of the mean for each value (and group) was less than ±1.5 per cent. Each value represented ten separate experiments for a concentration used. TSH and single injection of ACTH were given to 25 animals per group.

[2] These values were obtained in mice after prolonged administration of the following substances: ATP, pitressin, pitocin, growth hormone, follicular stimulating hormone, 11-dehydro-17-hydroxy-corticosterone-21-acetate, 17-hydroxy-corticosterone-21-acetate, desoxycorticosterone acetate, testosterone propionate, progesterone, estradiol benzoate, 21-acetoxy-pregnenolone. For concentrations used and the time of administration see the description of Methods. On the average 20 mice were used for each concentration of each substance.

(105 mg.) in the total acid-soluble phosphorus containing fraction; and 16.5% (53.5 mg.) in the nucleoprotein fraction.

Effects of the various substances on the content of the brain in the various phosphorus-fractions.—Prolonged administration of most of the substances used did not significantly modify the content of the brain in the various phosphorus containing fractions (Table 1). Prolonged administration of TSH resulted in a decrease of the content of the brain in the various phosphorus containing fractions. The decrease was statistically significant (average of 25 experiments). A temporary increase of the phospholipid content (average of 26%) followed a single intraperitoneal injection of ACTH. The changes observed were statistically significant (Table 1).

DISCUSSION

ACTH increases both the phospholipid content of the brain (Table 1) and the uptake in the brain of labeled phosphorus (Torda, 1953a). The results (Table 1) indicate that ACTH increases phospholipid synthesis and TSH decreases it. These changes were independent from the state of hydration due to these hormones.

Phospholipids of the brain are not fixed structural elements but may participate in the cerebral activity. The phospholipid content of the brain decreases during convulsions (Torda, 1953b) and after an injection of insulin (McGhee *et al.*, 1951). Furthermore, changes in the uptake of radioactive phosphorus in the brain phospholipid fraction occur during convulsions (Torda, 1953a) and during various experimental procedures (Dawson *et al.* 1950).

SUMMARY

1. The effects of various substances on the content of phospholipids, nucleoproteins, and total acid-soluble phosphorus compounds of the brain were ascertained.

2. Prolonged administration of TSH decreased the content in the brain of various fractions.

3. A single injection of ACTH induced a temporary increase of the phospholipid content of the brain. Prolonged administration of ACTH did not result in changes of the various phosphorus containing compounds of the brain.

4. The amounts of the various phosphorus containing fractions in the brain were not significantly modified by the other substances used (growth hormone, pitressin, pitocin, follicular stimulating hormone, desoxycorticosterone acetate, 11-dehydro-17-hydroxycorticosterone-21-acetate, 17-hydroxy-corticosterone-21-acetate, testosterone propionate, progesterone, estradiol benzoate, 21-acetoxy-pregnenolone, and ATP).

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INFLUENCE OF SULFHYDRYL COMPOUNDS ON *IN VITRO* STEROID PRODUCTION BY THE RAT ADRENAL GLAND[1]

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ALTHOUGH there have been numerous reports on the effects of adrenalectomy, adrenocorticotropic hormone and cortical steroids upon blood and tissue sulfhydryl levels (Binet and Poutonnet, 1943; Hess *et al.*, 1951; Goldzieher *et al.*, 1953a; Goldzieher *et al.*, 1953b), the effect of sulfhydryl and disulfide compounds upon corticoid output has been little studied. Carey *et al.* (1951) reported that large doses of glutathione depleted rat and guinea pig adrenal ascorbic acid and cholesterol. Bacq *et al.* (1953) demonstrated a large fall in adrenal ascorbate after administration of 2-aminoethanethiol (cysteamine) but no change in cholesterol content. Goldzieher and Edlin (1953) found a fall in urinary 17-keto and reducing steriods after prolonged treatment with cystine. The purpose of the present study was to observe the effect of a number of sulfur compounds and sulfhydryl-binding inhibitors upon the *in vitro* formaldehydogenic steroid output of rat adrenal glands.

METHODS AND MATERIALS

The animals used were 150 gm. female rats of the Sprague-Dawley strain. Quartered adrenal glands were incubated for two and one-half hours and the formaldehydogenic steroid content of the glands and medium was then determined in the manner reported in a previous paper (Hofmann and Davison, 1954). Substrates were present at the final concentrations stated, and were neutralized prior to adding them to the incubation medium.

Sodium acetoacetate was prepared by the method of Ljunggren (1924). The 2-aminoethanethiol (cysteamine) and 2,2′-dithio-bis-ethylamine (cystamine) were synthesized according to Mills and Bogert (1940). Coenzyme A was a preparation, 75% pure, by Pabst Laboratories. N-ethyl maleimide (NEM) was purchased from Schwarz Laboratories, Inc.

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RESULTS

Two sulfhydryl-binding inhibitors were incubated with the glands. N-ethyl maleimide (NEM) has been reported to be very specific for the sulfhydryl group, while iodoacetic acid will also interact with various other groups. Although the former proved to be a more potent inhibitor of formaldehydogenic steroid production, iodoacetate was also highly inhibitory at a concentration of 10^{-2} *M*, though without effect at 10^{-3}*M* (Table 1).

The effect upon steroid output of a number of sulfur-containing compounds was also determined. Three thiols exerted significant effects upon this system. Cysteine produced a highly significant depression while reduced glutathione gave a probably significant depression. On the other hand, cysteamine, the decarboxylation product of cysteine, produced a highly significant enhancement of steroidogenesis. These data are given in Table 1. Serine, used in the same concentration as cysteine, was without significant effect. The oxidized forms of the three thiols, cystine, oxidized glutathione and cystamine, were without effect. In view of the results of Klein and Lipmann (1953) who found a correlation in rat liver between Coenzyme A levels and acetate incorporation into cholesterol, a number of incubations were carried out with this coenzyme present at a concentration of fifty Lipmann units per flask. The substance did not affect steroid output, nor did the simultaneous addition of Coenzyme A and acetate at 10^{-3} *M* concentration.

TABLE 1. COMPOUNDS AFFECTING FORMALDEHYDOGENIC STEROID OUTPUT

Added compounds	Final conc. molar	No. of flasks	Net output μg. FS[1] per gram adrenals per 2.5 hours, ±S.E.[2]	x/c[3]	Fisher's "*p*"[4]
None	—	34	175 ± 12.7	—	—
NEM	10^{-3}	9	62 ± 24.0	0.35	<0.01
NEM	10^{-2}	4	0 ± 12.7	0.00	<0.01
Iodoacetate	10^{-2}	6	80 ± 29.3	0.46	<0.01
Cysteine	10^{-3}	7	114 ± 18.2	0.65	<0.01
Cysteamine	10^{-3}	7	261 ± 27.2	1.49	<0.01
Glutathione, Reduced	10^{-3}	6	106 ± 29.3	0.61	<0.05–>0.02

[1] FS—formaldehydogenic steroid, expressed as desoxycorticosterone.
[2] S.E.—standard error of the mean.
[3] Ratio to average of control values.
[4] Compared to average of control values.

As anticipated, the effect of N-ethyl maleimide could be overcome. These data are presented in Table 2. The inhibitor (NEM) was added ten minutes prior to the sulfur compounds in order to provide ample opportunity for its reaction. Incubations then proceeded as usual. Reduced glutathione re-

TABLE 2. REVERSAL OF INHIBITION BY N-ETHYL MALEIMIDE

Added compounds	Final conc. molar	No. of flasks	Net output μg. FS per gram adrenals per 2.5 hours ±S.E.	x/x'[1]	Fisher's "p"[2]
NEM	10^{-3}	9	62±24.0	—	—
NEM+Cysteine	10^{-3} 10^{-3}	7	42±28.6	0.68	>0.05
NEM+Glutathione, Reduced	10^{-3} 10^{-3}	7	193±31.7	3.11	<0.01
NEM+Cystine	10^{-3} 0.5×10^{-3}	5	9±32.4	0.15	0.2

[1] Ratio to average with NEM alone.
[2] Compared to average with NEM alone.

versed the inhibition by NEM, whereas cysteine and cystine had no such effect. Indeed it appears that cystine may actually have enhanced the inhibition.

In the hope of demonstrating a more profound or different effect upon a system which was functioning more actively, combinations of ACTH and sulfur compounds or NEM were used. Cysteine and reduced glutathione exhibited no effect in ACTH stimulated systems while cystine and NEM were inhibitory.

Finally interactions of cysteine and reduced glutathione were studied with three compounds, acetate, glycerol and acetoacetate, which had previously been shown to stimulate steroid synthesis (Hofmann and Davison, 1954). Here, for the first time a stimulatory effect was seen with glutathione. The combination of glutathione and glycerol greatly enhanced the net output above that seen with glycerol alone (Table 3). On the other hand, cysteine reduced the output to levels comparable to those observed with cysteine alone. Addition of either sulfhydryl compound to an acetate or acetoacetate-stimulated system also produced inhibition, of probable significance. The effect of glutathione appears to be very specific for a system stimulated by glycerol.

DISCUSSION

The inhibitory effects of the sulfhydryl-binding compounds on steroid output are not surprising. Such a complex system as steroid synthesis would almost certainly contain many enzymes dependent upon sulfhydryl groups for their activity. The concentration of N-ethyl maleimide producing partial inhibition (10^{-3} M) was several times greater than the concen-

TABLE 3. EFFECT OF ACTIVATORS AND SULFUR COMPOUNDS

Added compounds	Final conc. molar	No. of flasks	Net output μg. FS per gram adrenals per 2.5 hours, ±S.E.	x/x'[1]	Fisher's "p"[2]
Glycerol	10^{-2}	20	314 ± 38.4	—	—
Glycerol + Glutathione	10^{-2} 10^{-3}	10	451 ± 21.6	1.44	<0.01
Glycerol + Cysteine	10^{-2} 10^{-3}	8	107 ± 16.8	0.34	<0.01
Acetate	10^{-2}	8	236 ± 25.0	—	—
Acetate + Glutathione	10^{-2} 10^{-3}	8	155 ± 23.8	0.66	<0.05–>0.02
Acetate + Cysteine	10^{-2} 10^{-3}	8	102 ± 46.9	0.45	<0.05–>0.02
Acetoacetate	10^{-2}	16	471 ± 90.2	—	—
Acetoacetate + Glutathione	10^{-2} 10^{-3}	8	312 ± 23.0	0.66	0.01
Acetoacetate + Cysteine	10^{-2} 10^{-3}	6	246 ± 32.8	0.52	<0.05–>0.02

[1] Ratio to average with activator only.
[2] Compared to average with activator only.

tration of extracellular sulfhydryl in order to insure that ample amounts would reach the intracellular enzyme systems.

The effects of the sulfhydryl compounds are less easy to comprehend. If the inhibition by cysteine were simply due to the thiol group alone, perhaps through some oxidation-reduction effect, it might be expected that glutathione and cysteamine could inhibit as well as cysteine. From the results with serine and cysteamine it appears that both thiol and carboxyl groups are essential for the inhibition, but the nature of the inhibition is not clear.

The stimulation by cysteamine is interesting since its only known metabolic function is as a component of Coenzyme A. Kaplan and Lipmann (1948) report that, in the rat, the adrenal gland is second only to the liver in content of this coenzyme. The results obtained in our system are not necessarily conflicting, since the smaller molecule might pass the cell membrane and become part of Coenzyme A, while the phosphorylated coenzyme very probably cannot enter the cell.

The apparently specific effect of glutathione upon glycerol stimulation of steroid formation might be related to the report of Krimsky and Racker (1952) that it is the prosthetic group for glyceraldehyde-3-phosphate dehy-

drogenase. Glutathione might therefore be merely accelerating the entrance of glycerol into the metabolic pool.

SUMMARY

The sulfhydryl-binding compounds, N-ethyl maleimide and iodoacetic acid, have been shown to inhibit markedly formaldehydogenic steroid production by the rat adrenal gland. The inhibition by the maleimide is prevented by an equivalent amount of glutathione, but not by cysteine.

Cysteine and probably reduced glutathione also inhibit steroidogenesis, while the decarboxylation product of cysteine, cysteamine, stimulates steroid production. The disulfide forms of these three sulfhydryl compounds are without effect, as is Coenzyme A. The oxygen analog of cysteine, serine, does not affect steroid production.

In the presence of glycerol, glutathione further stimulates steroid formation whereas cysteine exhibits the usual inhibition. Both compounds act as inhibitors when either acetate or acetoacetate are present as substrate.

Acknowledgement

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POTENCY AND DURATION OF ACTION OF TRIIODOTHYRONINE AND THYROXINE IN RATS AND MICE

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RECENT studies have shown that 3,5,3′-L-triiodothyronine is three to five times as active as L-thyroxine by the goiter prevention method (Gross and Pitt-Rivers, 1952) and in increasing oxygen consumption in rats (Heming and Holtkamp, 1953). Gemmill (1953), however, found no significant difference in the potency of these two compounds by oxygen consumption studies in rats and anoxia sensitivity tests in mice. Oxygen consumption determinations in mice indicated that triiodothyronine was more potent than L-thyroxine and that the latent period and duration of action were similar (Maclagen, Sprott, and Wilkinson, 1952).

The present study was designed to compare the potency and duration of action of L-triiodothyronine and DL-thyroxine by the mouse anoxia sensitivity method of assay (Smith, Emmens, and Parkes, 1947) and by the effect on inhibition of release of I^{131} from the thyroid in rats. The release of I^{131} from the thyroid in rats is inhibited by treatment with thyroxine (Wolff, 1951) and the degree of inhibition is related to the dose of thyroxine administered (Perry, 1951).

METHODS

I. Male Sprague-Dawley rats weighing 165–240 gm. were provided with *Purina Fox Checkers* and tap water *ad libitum* unless otherwise noted. When propylthiouracil was used it was added to *Purina Mink Chow* at .03% concentration. Food was withheld for eight hours before and for twenty hours after I^{131} was administered. The rats were injected intraperitoneally with I^{131} and the radioactivity over the thyroid region of the neck was determined forty hours later (hereafter referred to as "zero time"). Counting was done with the rats lightly anesthetized with ether. The apparatus, procedure, and correction for nonthyroidal radioactivity were similar to those used by Wolff (1951). Daily counts were made at 24-hour intervals after "zero time" and were expressed as per cent of "zero time" counts after correction for physical decay of the isotope. The injections of DL-thyroxine and L-triiodothyronine (dissolved in 0.9% NaCl made slightly alkaline with NaOH) were made in a volume of 0.5 to 1.0 cc. by the intraperitoneal route.

II. Male white mice were given *Purina Fox Checkers* and tap water *ad libitum*. The DL-thyroxine and L-triiodothyronine were prepared as described above and injected intraperitoneally in a volume of 0.5 cc. Injections were made at appropriate intervals before asphyxiation, which was performed in the same room in which the animals were caged at a temperature of 23.5° C. The mice were placed individually in 950-cc. commer-

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cial canning jars and the lids were sealed. The survival time was computed from the time of sealing to cessation of respiration.

RESULTS

1. Fifteen rats were each injected with 25 μc. of I^{131} and the radioactivity over the thyroid determined forty hours later. The rats were then divided into five groups of three rats each and immediately injected respectively with 4 or 8 μg. of DL-thyroxine, or 0.8 or 1.6 μg. of triiodothyronine, the fifth group serving as controls. The diet was then changed to .03% propylthiouracil. Treatment with propylthiouracil blocks organic binding of iodine and thus increases the rate of loss of I^{131} from the thyroid without qualitatively affecting the inhibitory action of thyroxine (Wolff, 1951). A greater range in the degree of effect from various doses was thereby obtained. The injections of thyroxine or triiodothyronine were repeated at 24-hour intervals for a total of four doses. The daily loss of I^{131} from the thyroid was inversely proportional to the dose of thyroxine or triiodothyronine. All treated groups showed a slower rate of loss than the controls. The per cent of I^{131} remaining in the thyroid four days after "zero time" (24 hours after the last injection) was plotted for each group (average of three rats) against the log of the dose of thyroxine or triiodothyronine administered. Triiodothyronine appeared to be about ten times as potent as DL-thyroxine.

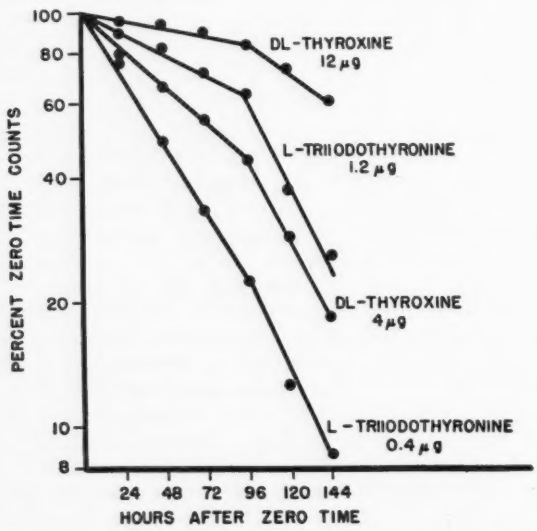

FIG. 1. Effect of various doses of thyroxine and triiodothyronine on release of I^{131} from thyroid in rats. Injections made at 0, 24, 48, and 72 hours after "zero time."

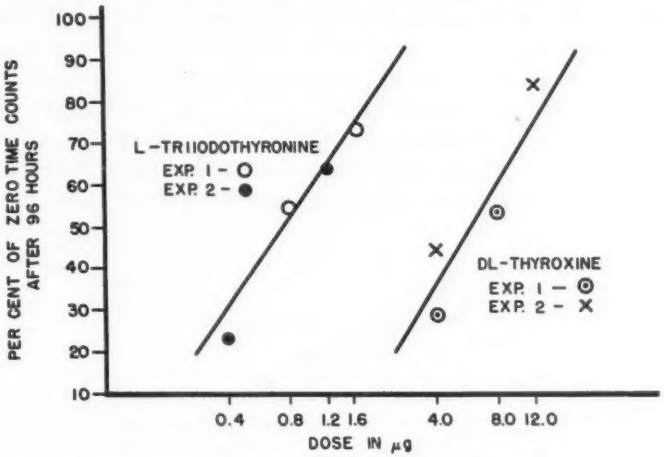

FIG. 2. Effect of various doses of thyroxine and triiodothyronine on I^{131} remaining in thyroid 24 hours after last of four daily doses.

2. Twenty rats were each injected with 25 μc. of I^{131} and the radioactivity over the thyroid determined forty hours later. Each rat then received 10 mg. of propylthiouracil (dissolved in an alkaline solution of 0.9% NaCl) by intraperitoneal injection, and the diet was changed to .03% propylthiouracil. The rats were then divided into four groups of five rats each and injected with 4 or 12 μg. of DL-thyroxine, or 0.4 or 1.2 μg. of triiodothyronine, respectively. The injections were repeated daily for a total of four doses. The daily loss of I^{131} from the thyroid was again inversely proportional to the dose of thyroxine or triiodothyronine administered (Fig. 1). The per cent of "zero time" counts remaining after four days (24 hours after the last injection) was plotted for each group against the log of the dose of thyroxine or triiodothyronine administered. Triiodothyronine appeared to be about six times as active as DL-thyroxine.

A log dose-response line plotted from the combined data from experiments 1 and 2 is shown in Figure 2. On the average triiodothyronine appeared to be about eight times as active as DL-thyroxine. After treatment was discontinued the loss of I^{131} was accelerated to a slightly greater degree in the rats treated with triiodothyronine than in those treated with DL-thyroxine although it is apparent that with both compounds larger doses had a more prolonged effect.

Effect of single dose on inhibition of release of I^{131}.—Eighteen rats were each injected with 35 μc. of I^{131} (without being fasted) and the thyroid radioactivity determined forty hours later. The rats were then divided into six groups of three rats each. Forty-eight hours after "zero time" single in-

jections of 10, 20, or 40 μg. of DL-thyroxine, or 2.5, 5.0, or 10.0 μg. of L-triiodothyronine were given. Loss of I^{131} from the thyroid was sharply inhibited for about 24 hours in the rats injected with 2.5 and 5.0 μg. of triiodothyronine and for about 48 hours in those injected with 10 μg. of triiodothyronine. The inhibition lasted for over 48 hours in the rats injected with thyroxine. The average for each group of three rats is shown in Figure 3. The magnitude of the dose affected the duration of the effect to some ex-

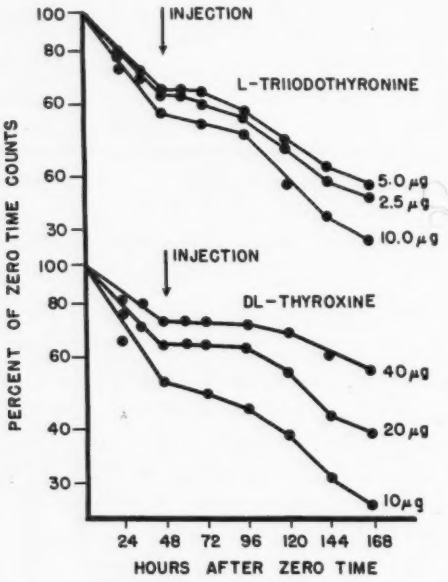

FIG. 3. Duration of effect of various single doses of thyroxine and triiodothyronine on inhibition of release of I^{131} from thyroid. Injections were made 48 hours after "zero time."

tent. However, in comparable dose ratios, 8:1, DL-thyroxine completely inhibited the release of I^{131} for at least 48 hours, while triiodothyronine completely inhibited for no longer than 24 hours.

Anoxia sensitivity tests in mice after daily doses.—Forty-five white mice averaging 20.5 gm. in weight were divided into five groups of nine mice each and were injected daily for five days with 0.25 μg. or 1.0 μg. of L-triiodothyronine, or 2.5 μg. or 10.0 μg. of DL-thyroxine, the last group being injected with 0.85% NaCl as controls. Twenty-four hours after the last injection the survival time was determined. The average for each group with the standard error was plotted against the log of the dose administered (Fig. 4). Although parallel dose-response lines were not obtained,

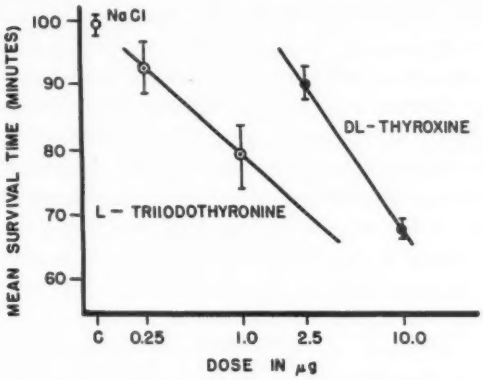

FIG. 4. Survival time of mice (anoxia test) 24 hours after five daily injections of various doses of thyroxine and triiodothyronine.

triiodothyronine appeared to be about five times as active as DL-thyroxine.

Duration of effect of single doses.—Seventy male white mice averaging 23.3 gm. in weight were divided into seven groups of ten mice each and injected once with 0.5 mg. of DL-thyroxine or 0.1 mg. of triiodothyronine at intervals of 24, 48, or 72 hours before asphyxiation. The control group received 0.5 cc. of 0.85% NaCl 24 hours before asphyxiation. The survival times

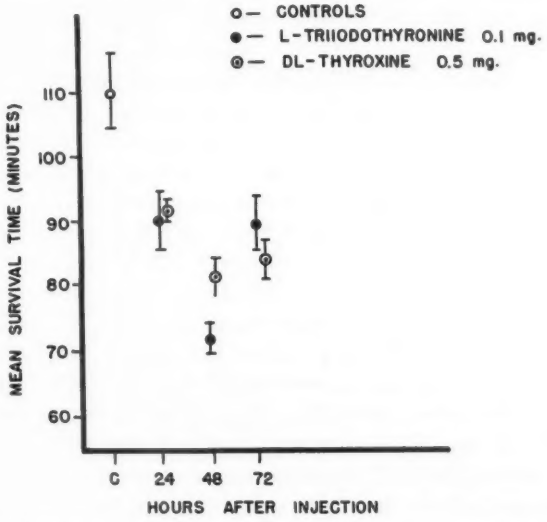

FIG. 5. Survival time of mice (anoxia test) after single injection of equivalent amounts of thyroxine or triiodothyronine given at various intervals before test.

(average for each group with standard errors) are shown in Figure 5. The survival times were similar in the groups injected 24 and 72 hours before the test; of the groups injected 48 hours before the test, those treated with triiodothyronine showed a greater effect than those treated with DL-thyroxine. Preliminary experiments, using fewer mice, showed no significant reduction in survival time in those injected 6 and 12 hours before asphyxiation. Experiments using smaller single doses of triiodothyronine or DL-thyroxine yielded inconclusive results, but suggested that thyroxine was active for four days following a single injection, while triiodothyronine was active for no longer than two days.

DISCUSSION

If it is assumed that DL-thyroxine possesses about half of the activity of L-thyroxine, the present finding that triiodothyronine is from five to ten times as active as DL-thyroxine on a weight basis agrees fairly well with the previously reported studies which indicated that triiodothyronine was from three to five times as active as L-thyroxine. The action of thyroxine in inhibiting the discharge from the thyroid of endogenous thyroxine is mediated through the pituitary by suppression of thyrotropin secretion and is thus similar to the mechanism of action of thyroxine in preventing goiter in rats fed thiouracil. It therefore appears logical that the relative potency of triiodothyronine and thyroxine as determined by these two methods would be in close agreement.

The finding that triiodothyronine is at least five times as active as DL-thyroxine in decreasing the survival times of mice in closed vessels suggests that triiodothyronine also has a more potent direct effect on metabolic processes than thyroxine, as had been shown by oxygen consumption studies. The findings of enhanced potency of triiodothyronine as compared with thyroxine and similar latent period and duration of action, as shown in the present study, agrees with the results obtained from oxygen consumption tests in mice (Maclagan, Sprott, and Wilkinson, 1952). The reason for the discrepancy between the present study and those of Gemmill (1953) is not apparent. Thyroxine did appear to have a more prolonged effect than triiodothyronine in suppressing the action of thyrotropin. This is compatible with the view that triiodothyronine may be more rapidly metabolized but does not necessarily imply that thyroxine is converted into triiodothyronine before becoming active. Recent studies involving serial measurements of the basal metabolic rate in myxedematous patients treated with thyroxine and triiodothyronine showed a qualitative difference in the responses which may indicate that the total calorigenic effects are similar for the two compounds (Blackburn *et al.*, 1953). The present

study would seem to support the view that triiodothyronine has a greater initial activity which is of shorter duration than that of thyroxine.

SUMMARY

The potency and duration of action of L-triiodothyronine and of DL-thyroxine were compared by determining their effects on the release of I^{131} from the thyroid in rats and on the sensitivity to anoxia in mice. Triiodothyronine appeared to be from five to ten times as active as DL-thyroxine by each method. No significant difference in latent period or duration of action was shown in mice following single injections and tested over a period of 72 hours. DL-thyroxine appeared to exert a more prolonged effect than triiodothyronine in suppressing the release of I^{131} from the thyroid in rats when doses of similar potency were compared.

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IN VITRO METABOLISM OF RAT MAMMARY GLAND: RESPIRATION, AEROBIC GLYCOLYSIS AND CARBON DIOXIDE PRODUCTION[1,2]

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THE mammary gland of the young virgin rat consists of a relatively simple group of ducts ramifying into a fatty pad. During recurring estrous cycles and the first part of pregnancy considerable proliferation (growth) of these ducts is observed. At about midpregnancy the growth of the lobule alveolar system is nearly completed. As pregnancy nears its termination the gland becomes a potential milk secreting (working) tissue however a limited secretion results in the accumulation of colostrum. At parturition, profuse secretion starts and its intensity may continue to increase for about $2\frac{1}{2}$ weeks (Brody and Nisbet, 1938) followed by a slow decline in secretory activity. The alveolar tissue regresses (involutes) following weaning. These physiological processes are known to be dependent upon intricate endocrine influences. The mammary gland is, therefore, a tissue which grows, works (secretes), and ages (involutes) in cycles throughout the reproductive life of the animal. The complexity of the tissue with its variable gross chemical composition throughout the physiological cycle demands judicius selection of the activity reference base, especially when one wishes to compare the various physiological states. Activity is expressed as units/hour/mg. nitrogen in these experiments. Special care was taken to minimize the retention of colostrum and milk. At least, the nitrogen reference base minimized the affect of varying proportions of fat. With data on the nucleic acids of this tissue, it has become more reasonable to use desoxyribonucleic acid as the activity reference base (Kirkham and Turner, 1953).

Kleiber, *et al.* (1943) studied the *in vitro* oxygen consumption of the rat mammary gland from animals pregnant 20 days and lactating 21 days, based upon various reference analysis, including nitrogen. Since these authors studied ". . .parts of the gland, excised with forceps. . ." it seems

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[2] Contribution from the Department of Dairy Husbandry, Mo. Agri. Exp. Station, Journal Series No. 1377. Approved by the director.

doubtful that the conditions of a true thin tissue slice were even closely approximated and therefore these data probably fail to reflect the true *in vitro* oxygen consumption. Numerous studies have been reported by Folley and co-workers (Folley and French, 1949a, 1949b, 1950) which utilize thin rat mammary gland slices. Their work clearly showed that in the presence of glucose, lactating rat tissue metabolized with an R.Q. greater than unity which confirms the earlier observations in this laboratory obtained by arterio-venous differences in the goat udder (Reineke, *et al.*, 1941). They also observed that lactating tissue had a higher respiration rate than was observed in rats pregnant 20 days. The Pasteur effect was marked. Other authors have claimed to work without the use of thin slices and "found replicates in good agreement" by use of small pieces of tissue cut from the mammary pad with scissors (Tuba, *et al.*, 1950). In their report the respiration rate on a dry weight basis of tissue from pregnant (10–14 days) and lactating (18–21 days) rats, the latter was 3 times as large as the former which is in agreement with Kleiber, *et al.* (1943) but in contrast to a ca. 8 fold increase observed by Folley and French (1949b).

In initiating studies on the metabolism and the role of the various enzyme systems in the growth and secretory activity of the mammary gland, it seemed desirable to follow the changes in repiration, aerobic glycolysis, and "metabolic" carbon dioxide production on true tissue slices *in vitro* throughout the entire normal reproductive cycle of the albino rat. From these data it should be possible to select metabolic reference points of maximum biological activities with reference to growth, secretory activity, and subsequent involution in the normal cycle and as an index of the production of comparable biological activity under experimental conditions involving one or more hormones.

METHODS AND MATERIALS

Virgin albino rats not in estrous and weighing between 150 to 170 gm. supplied the mammary tissue (inguinal and abdominal glands only) for the virgin state. Similar animals were bred to supply the glands in various stages of pregnancy and lactation. Only animals nursing more than 5 pups were used for the latter series. Involuted glands were obtained by removing 25 day old pups from their mother which was sacrificed 5 days later. Most data were obtained by the method of Summerson (1939) with 0.3% glucose as substrate in at least duplicate samples except for the virgins where the quantity of tissue was limited. About $\frac{1}{3}$ of the data were obtained by the method of Dixon and Keilin (Dixon, 1951). Since these measurements were at random throughout the various physiological conditions and since both methods seem to agree quite well these data were combined without designation. The gas phase was 95% O_2 and 5% CO_2; temperature 38°; initial pH 7.2. All tissues were sliced with the Stadie-Riggs microtome (Stadie and Riggs, 1944). Slices were shaken in cold Ringer-bicarbonate medium, blotted between filter paper, and again shaken in fresh medium which was followed by gentle blotting just prior to placing in the reaction flask. By use of true tissue

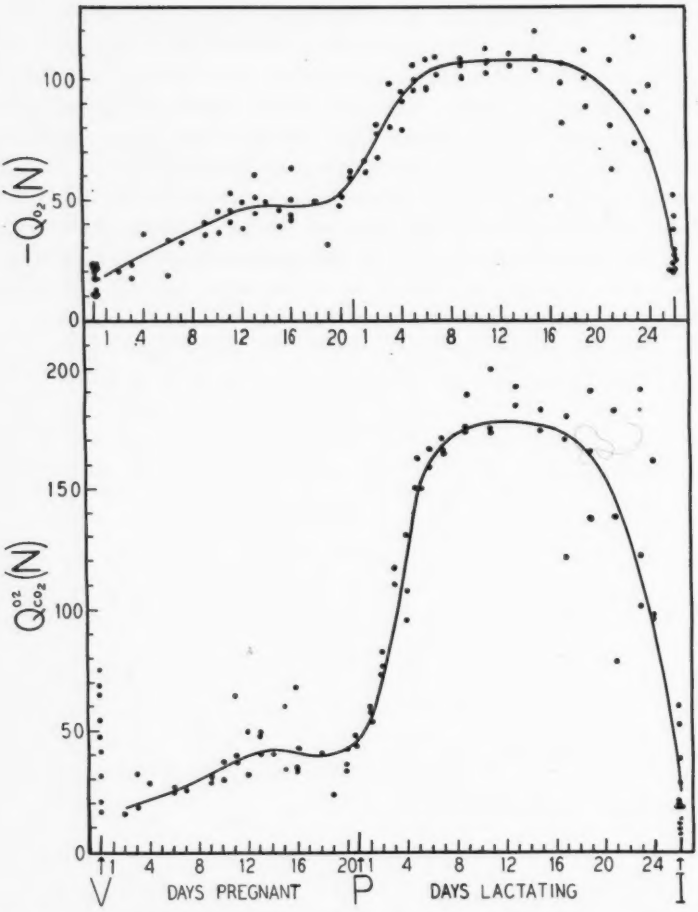

FIG. 1. Oxygen consumption and metabolic CO_2 production of rat mammary tissue. V represents data from virgin animals; I from post-lactating rats. P is the time of parturition.

slices, repeated leaching, and gentle blotting, it was hoped that the accumulation of colostrum in late pregnancy and milk throughout lactation was removed. A chemical test for reducing sugars in a saline medium containing slices was, following shaking at 38° for 1 hour, only very slightly but equally positive for tissue in mid-pregnancy, 20–21 days pregnant, and 13 days lactation. By using this standard procedure it is believed that these data, for the various physiological states, may be fairly comparable. Slices were removed from the metabolism flasks for colorimetric nitrogen determination at the end of the experiment (Umbreit, *et al.*, 1949). In all experiments the slices were allowed to metabolize for 30 minutes which resulted in an O_2 consumption in excess of

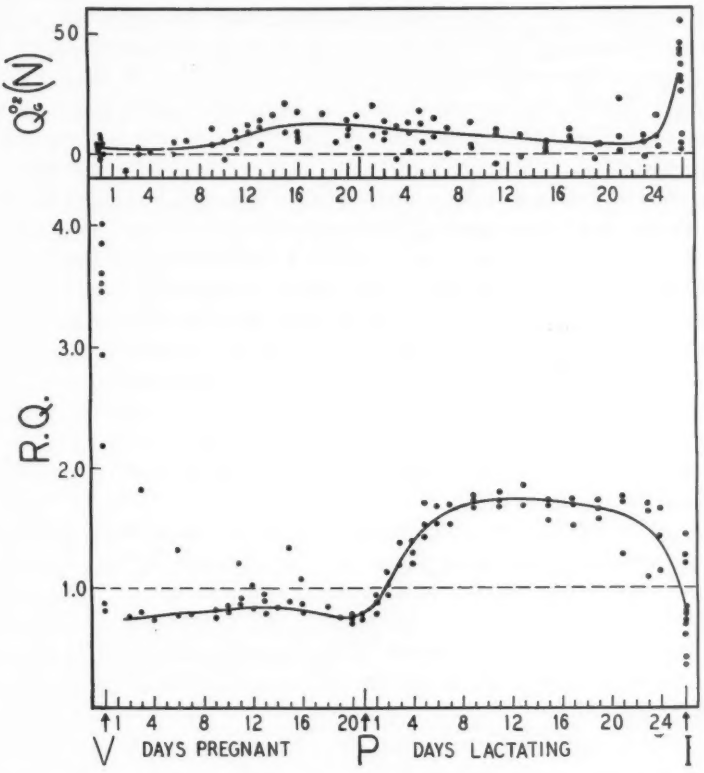

FIG. 2. Aerobic glycolysis (apparent) and R.Q. of rat mammary tissue. Abscissa same as Fig. 1.

50 μl. (80 to 150 μl. in most cases). The medium's pH did not decrease below 6.8 for the most active preparations during this interval.

Each point in Figures 1 and 2 represents the average of 2 or 3 measurements on tissue from one animal except those from virgins which represent one determination from one animal. The R.Q. was calculated from these averages. Data from an animal were discarded if the Q_{O_2} (N) did not agree within 10%.

RESULTS AND DISCUSSION

Since the R.Q. is independent of variable gross tissue composition more dependence may be placed on these values. Slices from virgin, non-estrous, animals gave in several cases a remarkably high R.Q. Here, the slices represent a trivial amount of epithelial cells, i.e., measurements were essentially made on adipose tissue in spite of attempts to trim off excess fat and slice only in the region of a nipple. The adipose nature of the gland con-

tinues to a gradually less extent throughout the first part of pregnancy. The R.Q. of mammae from pregnant animals is characteristically in the normal range below one, however, high values were still occasionally encountered. In these cases, values are averages of multiple determinations, therefore it is possible for one to observe an R.Q. above 1 in some mammae from pregnant rats (Fig. 2). There may be a tendency for the R.Q. to rise slightly during mid-pregnancy. Just prior to parturition values are close to 0.75. Within 2 days postpartum the quotient has reached 1.0 and is well on its way to a dramatic increase to about 1.7 at about the 8th day of lactation. As lactation proceeds values become less consistent but some remain high even at 24 days lactation. Since the rate of rat milk secretion gradually increases well beyond 9 days postpartum (Brody and Nisbet, 1938) when the R.Q. tends to plateau, there seems to be metabolic processes mediating the secretion of milk which are not reflected in the respiratory quotient (Fig. 2). Upon involution the quotient falls, in most cases to normal values but a few remain well above 1 and tissue from 2 animals had an R.Q. in the region of 0.4 (Fig. 2).

These data indicate that respiration increases about two-fold from the virgin state to mid and late-pregnancy. From about the 16th to 20th day of preganancy (a slight increase appears to occur just prior to parturition) to about the 7th day of lactation another $2\frac{1}{2}$ fold increase was observed which tended to be quite variable during late lactation. Upon involution O_2 consumption falls but not to the low values obtained from the virgin animals (Figure 1).

The "metabolic" CO_2 production from tissues of virgin animals were in general much greater than for early pregnant animals and had considerable variation over a wide range ($Q^{O_2}_{CO_2}(N) = 16$ to 75) (Fig. 1). Therefore, it is these values which are responsible for the extremely high R.Q. of mammary tissue from the virgin non-estrous rat. During pregnancy the CO_2 production in general follows the trend of respiration. However, following parturition it increases relatively more than respiration and results in high R.Q.'s within a few days post-partum. The trends in late lactation and involution conform to those of respiration but with a relatively greater drop in CO_2 production than O_2 consumption upon involution.

Aerobic glycolysis tends to be very low throughout under the conditions of this study with the exception of the involuted tissue where its CO_2 index tends to exceed the gas indicies for respiration and "metabolic" CO_2 production. There may be a slight tendency for higher rates during the latter part of pregnancy and the first part of lactation. It is entirely possible that this index for glycolysis includes acid formation other than lactic acid.

The R.Q. data are in good agreement with Folley and French's values of 0.83 for the 20th day of pregnancy, 1.0 for the first day of lactation, and

about 1.6 for the 8th to 15th days of lactation (glucose substrate) (Folley and French, 1949b). These authors suggest that the R.Q. above 1 during lactation reflects the net fat synthesis of the tissue but this interpretation has doubtful relation to the observation of the very high R.Q. of the virgin rat's mammary gland. Furthermore, the apparent aerobic glycolysis tended to be considerably lower relative to respiration than reported by Folley and French (1950). The rise of glycolysis in involuted tissue reflects a relative predominance of these processes over the respiratory system at a time of alveolar regression.

The more noticeable scattering of data from tissue taken from animals in late lactation and post-lactation is probably due to varying degrees of self weaning and suggests caution in metabolic work involving the use of mammary tissue from such animals.

SUMMARY

O_2 consumption, "metabolic" CO_2 production (R.Q. calculated), and aerobic glycolysis (apparent) have been studied as activity per mg. nitrogen of mammary tissue from virgin, pregnant, and post-lactating rats. O_2 consumption and CO_2 production increased as pregnancy progressed and increased more rapidly following parturition. The latter increased more rapidly than the former, therefore resulting in an R.Q. greater than 1 for lactating tissue. Several very high R.Q. values were observed in tissue from virgin, nonestrous animals. Aerobic glycolysis was quite low throughout the various physiological states except upon involution it tended to exceed respiration.

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STUDIES IN HISTOCHEMISTRY: EFFECTS OF STRESS CONDITIONS, ACTH, CORTISONE AND DESOXYCORTICOSTERONE ON THE QUANTITATIVE HISTOLOGICAL DISTRIBUTION OF ASCORBIC ACID IN ADRENAL GLANDS OF THE RAT AND MONKEY[1,2]

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INTRODUCTION

MEASUREMENTS of ascorbic acid in rat adrenals have been commonly employed as an indication of adrenal cortical function following treatments such as the imposition of stress conditions (Sayers *et al.*, 1945), or the administration of adrenocorticotropin (Sayers *et al.*, 1944, 1946), or crystalline steroid compounds, (Sayers and Sayers, 1947; Winter, 1950). In these studies the concentration of adrenal ascorbic acid rapidly diminished as the result of stress conditions or ACTH administration. Injection of cortisone or desoxycorticosterone 1 hour before exposure to stress prevented the depletion of adrenal ascorbic acid. On a milligram basis, cortisone was about four times as effective as desoxycorticosterone. However, daily administration of cortisone for six weeks resulted in a diminution of the concentration of ascorbic acid.

Most of these studies involved whole gland analyses which can only give a picture of the average of the changes in the separate zones of the adrenal. Local effects may have been lost or diminished by the pooling of material from all of the zones. It is not possible at present to determine the concentration of ascorbic acid in individual cells. However, a titrimetric method (Glick, 1935) for the analysis of ascorbic acid in single microtome sections was employed in an earlier investigation on fetal, postnatal, and adult bovine adrenal glands (Glick and Biskind, 1935, 1936). Recently, a spectrophotometric method was described by Glick *et al.*, 1953, for the analysis of ascorbic, dehydroascorbic and diketogulonic acids in microgram quantities

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[3] Data in this paper were included in a thesis submitted in partial fulfillment of the requirements of the Graduate School of the University of Minnesota for the degree of Doctor of Philosophy in Pathology.

of tissue, and a preceding study served to establish by this method the quantitative histological distribution of ascorbic acid in the adrenals of the normal rat and monkey (Bahn and Glick, 1954).

The present investigation was undertaken to extend this work to adrenals of the rat and monkey under conditions of stress and following the administration of ACTH, desoxycorticosterone or cortisone. The stress conditions imposed on the rat included chilling, inhalation of ether, and violent struggling. In addition, some of the rats were exposed to cold after treatment with cortisone or desoxycorticosterone. Monkeys were subjected to combined hypoxia and hyperthermia, and some of them were found to have granulomatous lesions which in themselves appeared to produce a state of stress.

Recent studies by Monier and Weiss (1952a, b) showed that the exposure of rats to cold or x-irradiation resulted in an increased urinary excretion of the initial oxidation products of ascorbic acid, dehydroascorbic and diketogulonic acids. Analyses of adrenal tissues for these two compounds were included in some of the present experiments in which rats were exposed to cold or forced to struggle violently.

MATERIALS AND METHODS

Monkeys

The adrenal glands from 24 monkeys (M. rhesus and M. cynomolgus) of both sexes were employed. The body weights ranged from 2–4 kg. The actual ages were unknown but judging from weight, gonadal development and dentition, the animals had not attained complete maturity. The monkeys were caged in an isolated room maintained at the usual laboratory temperature (70–80° F.). They were fed and handled as described previously (Bahn and Glick, 1954), and sacrificed by shooting in the head in a manner designed to minimize the stresses involved (Glick *et al.*, 1954). Five rhesus monkeys and 4 cynomolgus monkeys served as normal controls.

Infection.—In 5 rhesus monkeys granulomas of the lungs, liver and spleen were disclosed by post-mortem examination. Bacteriological culture and guinea pig innoculation of material obtained from the characteristic lesions in one of these animals established the presence of tubercle bacilli.

TABLE 1. PRETERMINAL TREATMENTS OF RHESUS MONKEYS
(From Zander, 1953, and Kubicek, 1953)

Monkey	Number exposures hypoxia	Number treatments hyperthermia		Interval between treatments (days)	Duration treatments (hours)	Min. $p.O_2$ (mm. Hg.)	Max. body temp. (°C.)
		Radiant heat	Pyrogens				
1	7	5	3	3–14	4½–7½	44–50	39.5–42.2
2	6	6	3	7–42	4½–8	46–58	39.5–43.2
3	12	0	0	1–9	½–1	50	—
4	3	3	2	3–42	4½–9½	38–58	39.5–42.7
5	12	0	0	1–5	½–1	50	—

TABLE 2. TREATMENT TERMINATING IN DEATH OF RHESUS MONKEYS
(From Zander, 1953, and Kubicek, 1953)

Monkey	Interval before terminal treatment	Duration terminal treatment	Terminal $p.O_2$ (mm. Hg.)	Terminal body temp. (° C.)
1	5 days	5 hours 22 min.	58	43.6
2	2 days	5 hours 8 min.	57	44.4
3	1 day	3 hours 32 min.	58	44.1
4	17 days	6 hours 20 min.	>150*	45.6
5	4 hours	3 hours 40 min.	54	43.1

* Received 100% oxygen during terminal experiment.

Hypoxia-hyperthermia.—The opportunity to study the animals which were subjected to a combination of hypoxia and hyperthermia was generously provided by Drs. W. G. Kubicek and H. A. Zander who employed them in independent investigations, (Kubicek, 1953; Zander, 1953). Prior to the terminal experiments which are reported in this paper, each member in a group of 5 monkeys was subjected repeatedly to hypoxia, and in 3 of the animals artificial fevers were also induced intermittently (Tables 1, 2). The intervals between the last nonfatal exposure to these stress conditions and the terminal experiments ranged from 4 hours to 17 days. In the final experiments which resulted in death after $3\frac{1}{2}$ to $6\frac{1}{2}$ hours of treatment, 4 animals (Nos. 1, 2, 3, 5, Tables 1, 2) were subjected to hypoxia by breathing oxygen-nitrogen mixtures in which the minimum partial pressure of oxygen was 54–58 mm. Hg., and, simultaneously, the body temperature of the 4 animals was raised artifically to 43.1–44.4° C. The fifth animal (No. 4, Tables 1, 2) was given 100% oxygen, and the terminal body temperature was elevated to 45.6°. Since the changes in adrenal ascorbic acid were practically the same in all 5 of the animals, the data will be presented for the group as a whole.

Adrenocorticotropin.—A single intraperitoneal injection of ACTH (Armour, 1 unit La-1-A standard/mg.) was administered to each of 2 rhesus and 3 cynomolgus monkeys. The rhesus monkeys received 15 mg. ACTH/kg. of body weight and the cynomolgus strain 30 mg./kg. One cynomolgus monkey was sacrificed 1 hour, and the remaining 4 monkeys 3 hours, after the injection.

Cortisone acetate.—Each of 2 cynomolgus monkeys received a single intramuscular injection of cortisone acetate (Merck, saline suspension, 10 mg./kg. body weight) and they were sacrificed 24 hours later.

Desoxycorticosterone acetate.—One cynomolgus monkey received a single intramuscular injection of desoxycorticosterone acetate (Ciba, in sesame oil, 10 mg./kg. body weight) and it was sacrificed 24 hours later.

Rats

Forty-one male albino rats (body weight 350–450 gm.) of the Holtzman strain were caged individually in an isolated room at 74 ±2° F. The rats were fed, handled and killed as described previously (Bahn and Glick, 1954). Ten rats served as normal controls.

Cold.—The effects of exposure to cold were studied in 17 rats which were placed in

individual cages in a refrigerator at 2° C. Three groups consisting of 5, 5, and 7 animals were sacrificed following exposure to cold for 20, 60, and 180 minutes, respectively.

Ether.—Three rats were exposed to ether vapor which caused death in 3–6 minutes.

Struggling.—Seven rats, unaccustomed to handling, were forced into a state of violent struggling by holding in the hand and compressing the thoracic and abdominal regions with the fingers. One group of 3, and another group of 4 rats were sacrificed after struggling for 2 and 10 minutes, respectively.

Adrenocorticotropin.—Each of 4 rats received a single intraperitoneal injection of ACTH (Armour, 1 unit La-1-A standard/mg.; 25–30 mg./kg. body weight). All were killed 1 hour later.

Cortisone acetate.—Each of 7 rats received cortisone acetate (Merck, saline suspension; 7 mg./kg. body weight) by a single subcutaneous injection. Four of these rats were sacrificed 24 hours following the injection. The remaining 3 rats continued to receive daily injections of the same dose for 3–4 weeks and were killed 24 hours following the last injection.

Cortisone acetate-cold.—Three rats received a single subcutaneous injection of cortisone acetate (7 mg./kg. body weight). Twenty-four hours later they were exposed to cold (2° C.) for 1 hour and then killed immediately.

Desoxycorticosterone acetate.—Each of 7 rats received desoxycorticosterone acetate (Ciba, in sesame oil; 7 mg./kg. body weight) by a single subcutaneous injection. Three of these rats were sacrificed 24 hours following the injection. The remaining 4 continued to receive daily injections of the same dose for 2½–3 weeks and were killed 24 hours following the last injection.

Desoxycorticosterone acetate-cold.—Three rats received a single subcutaneous injection of desoxycorticosterone acetate (7 mg./kg. body weight). Twenty-four hours later they were exposed to cold (2° C.) for 1 hour and then sacrificed.

Preparation of samples for histological examination and microchemical analysis.—The adrenal glands were removed as quickly as possible after death and frozen immediately on solid carbon dioxide. The equipment and procedures used for the histological examination and microchemical analysis of the tissue sections were identical with those described previously (Bahn and Glick, 1954; Glick *et al.*, 1953).

Presentation of data.—As described earlier (Bahn and Glick, 1954) a composite curve representing the ascorbic acid concentrations in the various histological zones of the adrenal gland of each species was constructed. The average quantity of ascorbic acid in each zone was estimated from the products of the width of the zone and its mean ascorbic acid content per section.

RESULTS

Monkeys

The composite curves of the distribution of ascorbic acid in the adrenals of the rhesus and cynomolgus monkeys are shown in Figure 1. It is apparent that for the rhesus animals all of the treatments employed and the infections caused some decrease in the concentrations. This loss was confined primarily to the regions having the greatest concentrations of ascorbic acid, the outer fasciculata and the reticularis. Changes of no appreciable significance occurred in the glomerulosa and medulla. The greatest effects

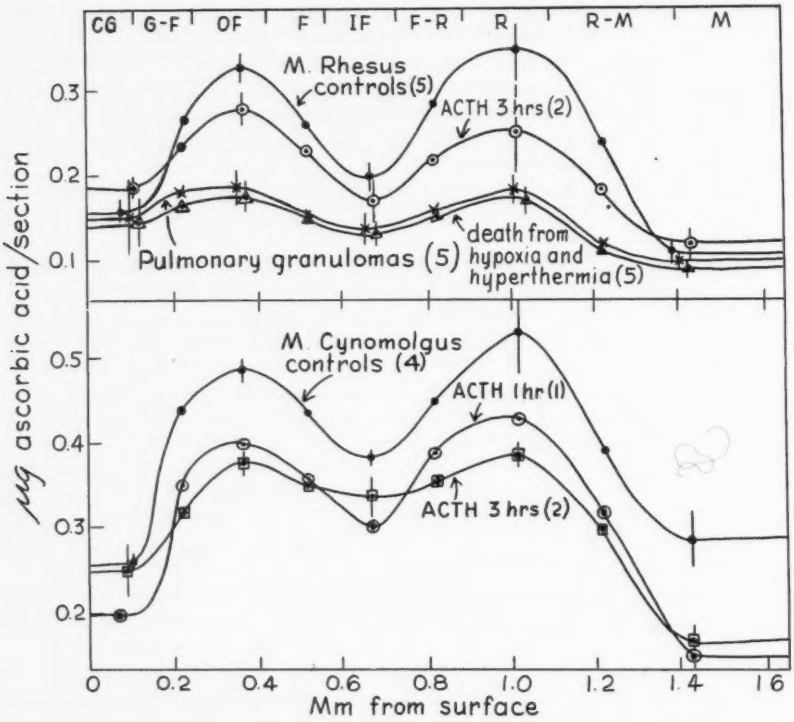

FIG. 1. *Upper*: Distribution of ascorbic acid in the adrenal gland of M. rhesus monkeys subjected to stress conditions or injected with ACTH (15 mg./kg. body weight). Microtome sections 4 mm. diameter, 16 μ thick (0.20 μl. vol.). For conversion of μg./section to mg./100 ml. tissue, multiply by 500. Points represent means, and vertical lines through points standard errors of means. *Lower*: Distribution of ascorbic acid in the adrenal gland of M. cynomolgus monkeys injected intraperitoneally with ACTH (30 mg./kg.). Microtome sections same size as above. For conversion of μg./section to mg./100 ml. tissue, multiply by 500.

occurred in the monkeys with granulomas or in those which died following exposure to simultaneous hypoxia and hyperthermia.

In rhesus monkeys the intraperitoneal administration of 15 mg. ACTH/kg. body weight did not cause as great a decrease in the adrenal ascorbic acid concentration as did the severe stresses. The losses of ascorbic acid in the cynomolgus monkeys 1 hour and 3 hours following a single intraperitoneal injection of ACTH were practically the same.

The effects of treatment of cynomolgus monkeys with cortisone or desoxycorticosterone are presented in Figure 2. The administration of a single intramuscular injection of cortisone 24 hours before sacrifice brought about

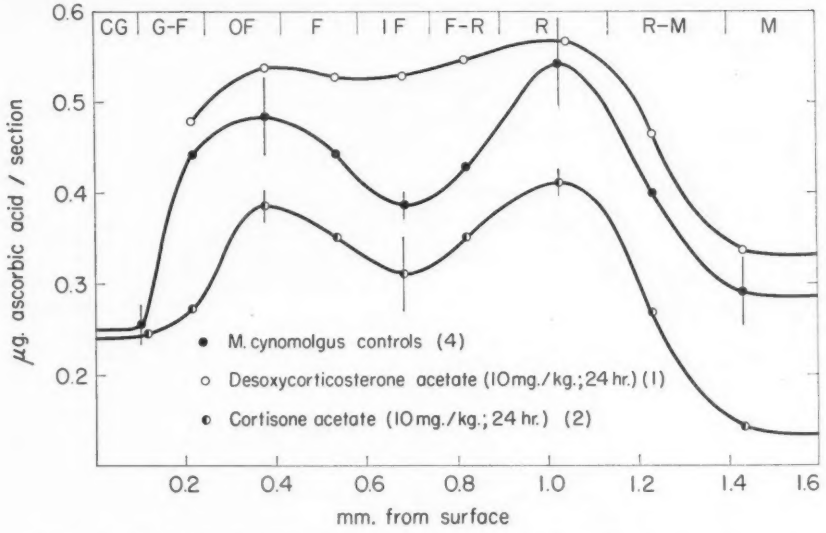

FIG. 2. Distribution of ascorbic acid in the adrenal glands of M. cynomolgus monkeys sacrificed 24 hours following the intramuscular administration of desoxycorticosterone acetate or cortisone acetate (10 mg./kg.). Microtome sections 4 mm. diameter, 16 μ thick (0.20 μl. vol.). For conversion of μg./section to mg./100 ml. tissue, multiply by 500.

a slight decrease in the concentration of ascorbic acid in the fasciculata and reticularis. Similarly administered desoxycorticosterone caused a small increase in the concentration of the ascorbic acid in the mid-fasciculata. Although, admittedly, these results on only a few animals are not highly significant, the changes are consistent with those found in the similar experiments on rats.

Rats

Figure 3 gives the composite curves of the distribution of ascorbic acid in rat adrenals. As in the monkey, all treatments caused a decrease in concentration, and the greatest losses were confined to the outer fasciculata and reticularis. No significant differences were detectable in the glomerulosa and medulla. Moderate decreases occurred following either a 2 or 10 minute period of violent struggling, death from the inhalation of ether, or a 20 minute exposure to a temperature of 2° C. A maximum loss following cold treatment was observed in the group of rats exposed to cold for 1 hour. After exposure to cold for 3 hours an increase in the ascorbic acid concentration occurred which might be interpreted to indicate some degree of acclimatization to the low temperature. The maximum decrease in ascorbic acid concentration following any of the treatments was observed in the

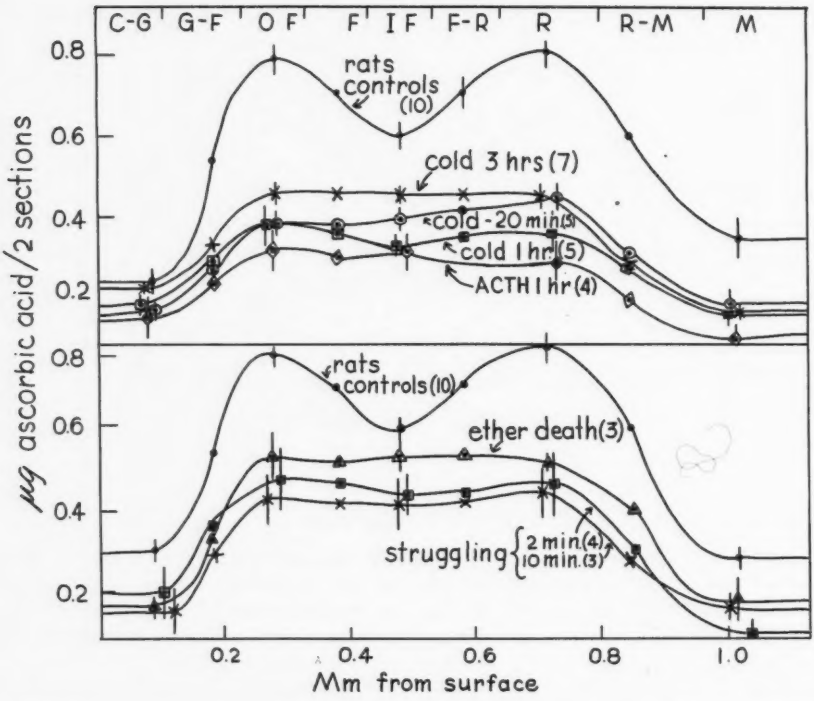

FIG. 3. *Upper*: Distribution of ascorbic acid in the adrenal gland of male albino rats exposed to cold (2° C.) or injected intraperitoneally with ACTH (25–30 mg./kg.). Microtome sections same size as above. For conversion of μg./2 sections to mg./100 ml. tissue, multiply by 1000. *Lower*: Distribution of ascorbic acid in the adrenal gland of male albino rats subjected to violent struggling or ether asphyxia. Microtome sections 2 mm. diameter. 16 μ thick (0.05 μl. vol.). For conversion of μg./2 sections to mg./100 ml. tissue, multiply by 1000.

adrenals from rats which received the single intraperitoneal injections of ACTH.

Table 3 gives the ratios of ascorbic acid to the sum of ascorbic, dehydroascorbic and diketogulonic acids in the adrenal zones of 2 normal and 6 stressed rats. From these data it is apparent that there was very little or no dehydroascorbic and diketogulonic acids in the cortex. The sum of the concentrations of dehydroascorbic and diketogulonic acids represented an appreciable fraction in the medulla, but only low concentrations of any of the substances occurred in this region.

The data reveal that significant losses of ascorbic acid were found only in the fascicular and reticular regions of the adrenal cortex, and a summary

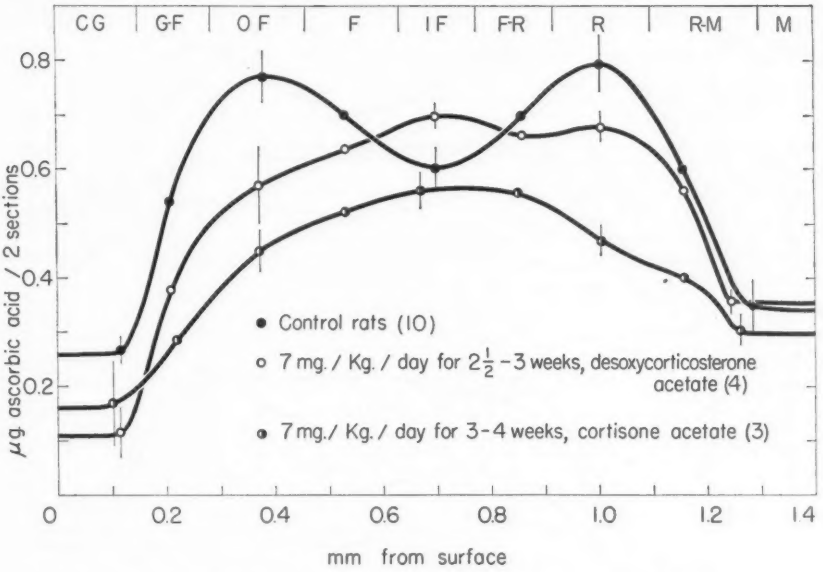

FIG. 4. Distribution of ascorbic acid in the adrenal gland of male albino rats injected subcutaneously with cortisone acetate or desoxycorticosterone acetate (7 mg./kg./day). Microtome sections 2 mm. diameter, 16 μ thick (0.05 μl. vol.). For conversion of μg./2 sections to mg./100 ml. tissue multiply by 1000.

of the relative decreases of ascorbic acid in each of these zones as a result of the treatments which were used is presented in Table 4.

Decreases in the ascorbic acid in the outer fasciculata and reticularis were observed when the animals were killed 24 hours after receiving the last of a series of daily subcutaneous injections of cortisone for 3-4 weeks (Fig. 4). The administration of a single subcutaneous dose of cortisone 24 hours before sacrifice brought about a moderate decrease in the concentration of ascorbic acid in the fasciculata (Fig. 5).

The subcutaneous administration of a single dose of desoxycorticosterone

TABLE 3. THE RATIO OF THE AMOUNT OF ASCORBIC ACID TO THE SUM OF THE ASCORBIC, DEHYDROASCORBIC AND DIKETOGULONIC ACIDS IN THE HISTOLOGICAL REGIONS OF THE ADRENAL GLAND OF THE RAT SUBJECTED TO STRESS CONDITIONS

Adrenal zone	Controls		Struggling 10 min.		2° C. 20 min.		2° C. 60 min.	
Rat No.	A	B	C	D	E	F	G	H
Fasciculata	1.0	1.0	0.9	1.0	0.9	1.0	1.0	1.0
Reticularis	1.1	1.0	0.7	0.9	0.9	1.0	1.0	0.8
Medulla	0.6	0.6	0.6		0.7		0.8	0.7

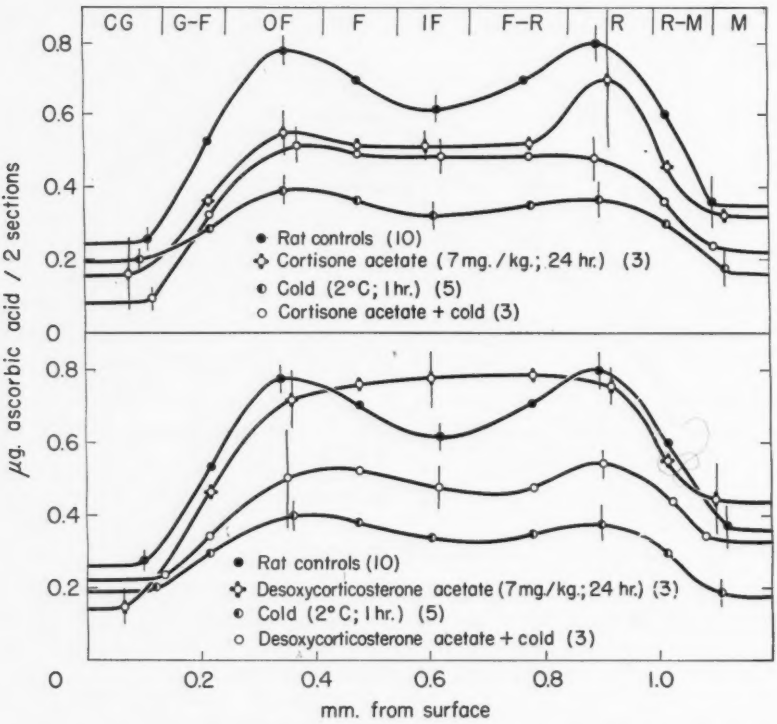

FIG. 5. Distribution of ascorbic acid in the adrenal gland of male albino rats given a single subcutaneous injection cortisone acetate (7 mg./kg.) or desoxycorticosterone acetate (7 mg./kg.) and/or exposed to cold (2° C.) for 1 hour. Microtome sections 2 mm. diameter, 16 μ thick (0.05 μl. vol.). For conversion of μg./2 sections to mg./100 ml. tissue multiply by 1000.

24 hours before sacrifice brought about a small increase in the concentration of ascorbic acid in the mid-fasciculata (Fig. 5). A small decrease in the outer fasciculata occurred when the rats were killed 24 hours after the last of a series of daily subcutaneous injections of desoxycorticosterone for $2\frac{1}{2}$–3 weeks.

The results of experiments on rats given a single subcutaneous injection of either cortisone or desoxycorticosterone 24 hours before exposure to cold for 1 hour is given in Figure 5. It is apparent that no appreciable changes in adrenal ascorbic acid occurred as a result of exposure to cold in those animals pretreated with cortisone. However, definite decreases in the fasciculata and reticularis occurred in the chilled rats which had been treated with desoxycorticosterone. It should be noted that, when the rats were pre-

TABLE 4. EFFECTS OF STRESS CONDITIONS AND ACTH ON THE AMOUNT OF ASCORBIC ACID IN VARIOUS ADRENAL ZONES

Animals			Condition or treatment		% decrease from controls	
Species	No. experimental	No. controls	Nature	Duration	Fasciculata	Reticularis
M. rhesus monkey	5	5	lethal hypoxia and hyperthermia	3–6 hr.	48	49
monkey	5	5	pulmonary granulomas	probably several months	48	49
monkey	2	5	ACTH 15 mg./kg. (intraperitoneally)	3 hr.	11	29
M. cynomolgus monkey	1	4	ACTH 30 mg./kg. (intraperitoneally)	1 hr.	18	20
monkey	2	4	ACTH 30 mg./kg. (intraperitoneally)	3 hr.	18	27
rat	5	10	cold (2° C.)	20 min.	44	42
rat	5	10	cold (2° C.)	60 min.	47	41
rat	7	10	cold (2° C.)	180 min.	30	32
rat	3	10	ether	3–6 min.	24	34
rat	4	10	violent struggling	2 min.	34	42
rat	3	10	violent struggling	10 min.	40	44
rat	4	10	ACTH 25–30 mg./kg. (intraperitoneally)	60 min.	56	63

treated with these steroids, the concentrations of ascorbic acid in the fasciculata and reticularis did not drop to the levels found when the animals were exposed to the same cold treatment without the administration of the steroids.

DISCUSSION

In a previous paper (Bahn and Glick, 1954) it was shown that there is a qualitative similarity in the histological distribution of ascorbic acid in the adrenal glands of the normal monkey and rat. The highest concentrations of ascorbic acid occurred in the outer fascicular and reticular zones. It is evident from the present data on stressed animals of both species that, regardless of the specific stimulus, the depletion of adrenal ascorbic acid occurred principally in the outer fasciculata and the reticularis and the quantitative responses to the stresses were roughly proportional to their severity and duration.

The qualitative changes brought about by ACTH were similar to those observed following the imposition of stress conditions. In the rat, the quantitative changes in the concentration of adrenal ascorbic acid were as great as those caused by any of the stress conditions. In the monkey, however, the losses accompanying ACTH administration were less than those caused by the stress conditions. Moreover, the relative losses of ascorbic

acid following the intraperitoneal administration of ACTH to the rat were greater than those brought about by a comparable dose similarly administered to cynomolgus monkeys.

It was demonstrated by Vogt (1953) and Long (1947) that the ascorbic acid of the whole rat adrenal is depleted very rapidly by severe emotional stimuli or inhalation anesthesia. Gray and Munson (1950), Farrell and McCann (1952) and Snydor (1953) also showed that significant quantities of ACTH can be released into the blood within 1 minute following the onset of a condition of stress, and Bates (1953) observed that maximum adrenal ascorbic acid depletion occurred in approximately 10 minutes after a single intravenous injection of 1 μg. or less of ACTH. It was observed in this investigation that decreased concentrations of ascorbic acid resulted within 2 and 10 minutes following violent struggling, or after ether inhalation for a few minutes.

In the adrenals of both normal and stressed rats no significant accumulation of dehydroascorbic or diketogulonic acids was detected. If, during a state of stress, the pathway by which ascorbic acid is lost does proceed by way of oxidation to dehydroascorbic and diketogulonic acids, either these products are removed rapidly or they are converted quickly to other compounds, or both.

Desoxycorticosterone caused little change in the histological distribution of ascorbic acid in the adrenals of rats and monkeys 24 hours after a single dose of 7-10 mg./kg., nor did significant changes occur when the same daily dose of the hormone was administered to rats for approximately 3 weeks. On the other hand, cortisone caused a decrease in the ascorbic acid of the fasciculata and reticularis of rats and monkeys 24 hours after a single injection of 7–10 mg./kg. and similar effects were observed when the same daily dose was given to rats for 3–4 weeks.

Pretreatment of rats with desoxycorticosterone or cortisone before exposure to cold resulted in inhibition of the loss of ascorbic acid which occurred in the fascicular and reticular zones of the adrenals from animals not given the pretreatment. The difference between the loss produced by desoxycorticosterone alone and that effected by following the hormone treatment with exposure to cold was appreciable. However, no difference was apparent between the loss caused by cortisone alone and that resulting from the cortisone administration followed by the cold exposure.

SUMMARY

The quantitative histological distribution of adrenal ascorbic acid was studied in rats and monkeys following treatments with ACTH, cortisone, or desoxycorticosterone, or exposure to the stress conditions of chilling, violent struggling, ether anesthesia, and combined hypoxia and hyper-

thermia. The adrenals of monkeys which had granulomatous lesions in the lungs and other organs were also investigated. All of the stress conditions caused a decrease in the concentration of ascorbic acid, chiefly in the outer fascicular and reticular zones.

In both rats and monkeys the responses to intraperitoneal injections of ACTH were qualitatively similar to those effected by stress conditions. The magnitude of the loss of adrenal ascorbic acid after ACTH treatment of rats (25–30 mg./kg. intraperitoneally) was of the same order as that caused by cold exposure (2° C.) for 1 hour. In monkeys the loss produced by comparable intraperitoneal doses of ACTH was less than that caused by the stress conditions.

Little change in the concentration of adrenal ascorbic acid of rats and monkeys was observed in animals sacrificed 24 hours following a single subcutaneous or intramuscular injection of desoxycorticosterone acetate (7–10 mg./kg.). When this dose of the hormone was administered daily to rats for $2\frac{1}{2}$–3 weeks, a small decrease in the concentration of ascorbic acid occurred in the outer fasciculata.

Moderate decreases in the adrenal ascorbic acid concentrations occurred in both the rats and monkeys when they were sacrificed 24 hours after a single injection of cortisone acetate (7 mg./kg. subcutaneously in rats, 10 mg./kg. intramuscularly in monkeys). When this dose was administered daily for 3–4 weeks to rats, distinct decreases in the concentration of ascorbic acid were observed in the outer fascicular and reticular zones. When the rat was given a single subcutaneous dose 24 hours before exposure to cold (2° C.) for 1 hour, both cortisone acetate and desoxycorticosterone acetate inhibited the losses of ascorbic acid.

Little or no dehydroascorbic and diketogulonic acids were found in any of the zones of the adrenal from normal rats or those exposed to cold or forced to struggle violently.

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LUTEOTROPHIC FUNCTION OF AUTOGRAFTS OF THE RAT HYPOPHYSIS[1]

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WESTMAN and Jacobsohn (1938) in experiments with hypophyseal stalk-sections in rats, reported certain results that have been difficult to explain. Adult, cyclic females were operated on during estrus or proestrus by the authors' usual technique. This included the placing of a barrier of metal foil between the cut end of the stalk and the hypophyseal capsule (Jacobsohn, personal communication). Although the intended purpose of the barrier was to prevent regeneration of nerve fibers, regeneration of the hypophyseal portal veins must have been equally impossible. Control animals were totally hypophysectomized. In all cases the cervix uteri was electrically stimulated 2 to 5 hours *after operation*. The uteri were traumatized 5 or 6 days later and were subsequently examined for deciduomata. Whereas the 7 completely hypophysectomized animals were negative, the 5 stalk-sectioned rats were positive. With the stalk having been cut before the time of cervical stimulation, how could the latter have induced pseudopregnancy, if this involves a reflex that is analogous to the ovulation reflex of the rabbit? It is important to note that Westman and Jacobsohn did not include control experiments in which the stalks were cut and cervical stimulation was omitted.

Recently Desclin (1950) described experiments in which he transplanted anterior hypophyses of estrogenized adult female rats to the kidney, obtaining luteotrophic function of the grafts in the absence of any fragments of gland remaining in the original site. In this instance the test for corpus luteum function was vaginal mucification in the presence of amounts of estrogen sufficient to cause vaginal cornification in the hypophysectomized controls. Desclin's interpretation is that estrogen can cause luteotrophin secretion by direct action on the hypophyseal tissue; that this does not require integrity of the connection of the hypophysis with the hypothalamus. No control experiments were reported that would test the possible luteotrophic activity of grafted hypophyses in the absence of estrogen.

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The present investigation demonstrates that anterior hypophyses, removed from female rats shortly after ovulation and transplanted to the renal capsule, will maintain fully functional corpora lutea without any stimulus other than may have resulted from the operation itself.

MATERIALS AND METHODS

Thirty adult, cyclic, virgin rats of the Vanderbilt strain were employed in these experiments, ranging in age from 5 to 11 months. Vaginal smear histories were recorded for several weeks preceding operation. The time of ovulation in the current cycle was judged on the basis of regularity of the preceding cycles (Everett, 1948; Everett, Sawyer and Markee, 1949; Everett and Sawyer, 1949). All operations were performed on the day after presumptive ovulation, when the vaginal smear registered full cornification.

Hypophysectomy was accomplished by the parapharyngeal route under ether anesthesia. To minimize fluid in the respiratory passages and resulting obstruction of the tracheal cannula,[2] atropine sulfate (10 mg./kg. body weight, in physiological saline)[3] was injected subcutaneously about 10 minutes before induction of anesthesia. The hypophysis was removed by suction and was recovered by a trap in the vacuum line.

In most of the transplantation experiments surgical approach to the left kidney was performed at the very outset, the wound then being temporarily closed until hypophysectomy had been accomplished. The pars distalis, usually in 2 pieces, was separated from the posterior lobe and inserted into a pocket inside the renal capsule. Pressure of the perirenal fat served to hold the graft in place as the body wall was being repaired. In 2 cases transplantation of pars distalis was made to a fascial pocket postero-lateral to the left common carotid artery.

Four days after transplantation or simple hypophysectomy the uterus was traumatized at laparotomy (ether anesthesia). Two silk threads were looped through short segments of the left uterus and tied along the antimesometrial border.

Eight days after the initial operation, hence 4 days after uterine traumatization, the uteri were examined for gross evidence of deciduomata. The degree of response was estimated on a scale of four. Portions of the traumatized uterine segments and at least one ovary were fixed in Zenker's fluid for histological examination. The cranium was opened, the brain was removed and a portion of the cranial floor including the pituitary capsule was placed in Heidenhain's Susa fixative for 24 hours. Decalcification was completed in 5% trichloracetic acid for 48 to 72 hours, after which washing and dehydration were accomplished in successive changes of 95% alcohol and dioxane. Serial sections were then prepared and rigorously searched for remnants of the hypophysis.

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[2] The tracheal cannula that proved most satisfactory is a 10 cm. length of polyethylene tubing (1.57×2.08 mm. inside and outside diameters, resp., Clay-Adams #PE205). The tube is beveled at the tip to aid its passage through the glottis by way of the oral cavity and pharynx. A retractable soft wire core maintains a slight curve of the beveled end of the tube during its introduction, and is then removed. A stiff rubber washer, placed along the tube about 4 cm. from the tip is caught by the upper incisor teeth to prevent accidental retraction during manipulation of the trachea and pharynx.

[3] This amount of atropine is far below the minimal subcutaneous dose (230 mg./kg.) effective in blockade of the ovulatory stimulation of the rat hypophysis (Everett and Sawyer, 1953).

RESULTS AND DISCUSSION

The result are summarized in Table 1. The definitive control and experimental groups (I and II) comprise animals in which complete removal of pars distalis had been accomplished. The inclusion of additional groups in which hypophysectomy had been incomplete requires explanation. Inadequate vacuum during the early stages of the investigation resulted in a surprisingly large number of such cases. Where extensive fragments of retained pars distalis were found in the control series the data are included because the results bear on the question at issue (group V). The least amount found in this group (in the negative case) was a single fragment measuring approximately $1.0 \times 0.5 \times 0.2$ mm. Groups III and IV retained considerably less. In control group III the largest amount was a single piece approximately $0.55 \times 0.5 \times 0.1$ mm. One other rat had almost as much, while the other four had much less. Group IV was purposely limited to grafted animals in every one of which the aggregate of retained bits was obviously far less than the largest amount in group III.

Inspection of the table will disclose that every hypophysectomized animal that bore a graft of pars distalis developed a strong deciduoma reaction in the traumatized uteri. Among the controls deciduomata were absent in group I in which hypophysectomy had been complete, as well as in group III in which minute fragments of anterior lobe remained *in situ*. Retention of a large fragment, however, was nearly always sufficient in itself to produce deciduomata that were often of maximal size (group V).

The corpora lutea of all the animals that developed deciduomata were distinctly larger than in the negative cases, resembling those of normal pseudopregnancy histologically. On the other hand, examination of serial sections of the ovaries discloses no evident difference between grafted animals and controls with respect to status of follicles and interstitial tissue. No healthy follicles are found beyond the stage of incipient antrum formation. In this respect the ovarian picture is decidely at variance with that of

TABLE 1. DECIDUOMA FORMATION IN FEMALE RATS BEARING AUTOGRAFTS OF ANTERIOR HYPOPHYSIS

Group no.	Hypophysectomy	Grafted to:	No. of rats	Deciduoma reaction				
				++	+++	++++	Total pos.	Total neg.
I	Complete	—	3				0	3
II	Complete	Renal capsule	6		1	5	6	0
III	Nearly complete	—	6				0	6
IV	Nearly complete	Renal capsule	6		2	4	6	0
		Neck	2		1	1	2	0
V	Extensive fragment left	—	7	2	1	3	6	1

normal pseudopregnancy, where prominent vesicular follicles are common. It is also significant that wide spread wheel-cell formation appears in the interstitial tissue of both the grafted and the control animals. Thus, neither the grafts (groups II and IV) nor the retained fragments of anterior lobe (group V) had been secreting effective amounts of either FSH or LH. They had been releasing sufficient luteotrophin, however, to maintain corpus luteum function.

The grafts were uniformly well vascularized, as attested in the fresh tissue by their normal pink color. In the histological sections the sinusoids are seen to be well-developed; the parenchyma remains in good condition save in the interiors of the grafts, where it has been replaced by connective tissue. Characteristically, healthy parenchyma extends inward from all surfaces to a depth of some 0.1–0.2 mm., while the dimensions of the connective tissue core vary with form of the graft. The inner regions must have been severely damaged before circulation became reestablished.

It must be assumed that for some ill-defined period of time after transplantation hormones already present were passed into circulation by absorption from necrotic tissue. In fact, the question arises whether maintenance of corpora lutea throughout the 8-day period may have been produced by such absorption rather than by actual secretion of luteotrophin. Several considerations, however, favor the latter alternative. According to current views, the hypophyseal luteotrophic factor in rats is the lactogenic hormone (Evans *et al.*, 1941; Tobin, 1942; Nelson and Pichette, 1943; Everett, 1944; Gaarenstroom and de Jongh, 1946; Desclin, 1948; Mayer, 1951). In substitution experiments the required amounts of lactogen for maintaining pseudopregnancy are on the order of 15 to 20 I.U. injected subcutaneously once daily. According to Reece and Turner (1937) the average lactogen content of group of estrous rat hypophyses was 4.8 bird units per gland, equivalent to approximately 0.2 I.U. (1 Reece-Turner unit = ca. 0.045 I.U.—Meites *et al.*, 1941). One must admit, of course, that minute amounts released gradually and continuously may be far more effective than a large amount injected once daily. Yet the maturity of the connective tissue in the internal portion of the grafts indicates that deterioration of parenchyma and its removal had been precipitous rather than gradual. Such would be expected *a priori*. Thus it seems almost certain that corpus luteum function in the grafted animals reflected actual luteotrophin secretion.

Conclusive evidence is furnished by certain additional experiments that will be fully described in a subsequent publication. These are long-term experiments that demonstrate corpus luteum secretory function for 33, 60 and even 90 days after transplantation of pars distalis to the renal capsule.

There is no evidence of the formation of additional corpora lutea. Follicular apparatus and interstitial tissue remain atrophic.

It should be emphasized that in none of the experiments just mentioned or those listed in Table 1 was there any special stimulation administered that was designed to cause pseudopregnancy. It seems that the mere fact of removal of the gland from its normal relation with the hypothalamus favors luteotrophin secretion at the expense of the other gonadotrophic factors.

In this connection it is significant that pseudopregnancy was obtained in most of the control animals (group V) in which a large fragment of anterior lobe remained in place. These experiments are analogous to the stalk-sectioned series of Westman and Jacobsohn (*loc. cit.*). There now seems little doubt that the additional factor of cervical stimulation in their animals was superfluous. The fact of stalk-section itself was apparently the cause of pseudopregnancy.

Some readers may insist that irritation of the hypophysis during either the act of cutting the stalk or the act of transplantation is sufficient to account for this luteotrophic function. This hardly seems likely in view of the great length of time during which the new activity can be maintained. To this writer it seems the better choice to regard the effect as a release phenomenon. It is interesting that Greep and Jones (1950), considering possible explanations of the production of pseudopregnancy in rats by estrogen treatment, suggested that the fundamental action of estrogen here may be suppression of FSH and LH secretion, after which luetotrophin secretion "proceeds apace."

Desclin's interpretation of his results, to the effect that estrogen invoked luteotrophin secretion by direct action on the grafted hypophyses, does not now appear to be correct. The estrogen was superfluous. Nevertheless, the present experiments amply confirm his original observation of the fact that the rat hypophysis, although isolated from the central nervous system by transplantation to the kidney, can yet secrete luteotrophic hormone. The new information is that secretion of this particular hormone is actually favored by such isolation. In addition the present results emphasize sharply the physiological distinction in this species between the two gonadotrophic hormones FSH and LH, on the one hand, and the luteotrophic factor, on the other.

SUMMARY

Adult virgin female rats were hypophysectomized on the day following ovulation. In the experimental animals the anterior hypophysis was autotransplanted to the renal capsule (12 cases) or the vicinity of the common carotid artery (2 cases). Control animals were simply hypophysectomized.

The uteri of all rats were traumatized 4 day later and autopsy was performed on the eighth day.

None of the 9 control rats in which hypophysectomy had been either complete or nearly complete formed deciduomata. However, good deciduomata were formed in 6 grafted animals that had been completely hypophysectomized and 8 in which hypophysectomy had been nearly complete. In addition, 6 of 7 partially hypophysectomized, non-grafted animals that retained large fragments of the gland gave significant deciduoma reactions.

The results indicate that removal of the gland from its normal relationship with the hypothalamus, or even local interference with such relationship, not only allows luteotrophin secretion to take place, but actually favors this activity.

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THE ANTIDIURETIC ACTIVITY OF THE PLASMA OF ADRENALECTOMIZED, HYPOPHYSECTOMIZED AND ADRENALECTOMIZED-HYPOPHYSECTOMIZED RATS[1,2]

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DAMAGE of the supraoptic nuclei, the paraventricular nuclei, the supraoptico-hypophyseal tract or the neurohypophysis results in the development of diabetes insipidus (Fisher, Ingram and Ranson, 1938; Smith, 1951). Accordingly, the secretion of the antidiuretic hormone (ADH) appears to be dependent upon the integrity of the supraoptico-hypophyseal system. Since adrenalectomy results in an increased reabsorption of water by the renal tubules and a diminished diuretic response to a water load, it has been postulated that the secretion of the antidiuretic hormone is dependent also upon the integrity of the adrenal cortex (Gaunt, Birnie and Eversole, 1949).

Direct evidence in support of the aforementioned was not available until Birnie, Jenkins, Eversole and Gaunt (1949) demonstrated that the antidiuretic activity of the serum of rats is elevated after adrenalectomy. Although the procedure employed by Birnie *et al.* (1949, 1950) undoubtedly permits the detection of antidiuretic substances, the significance of their findings was questioned by studies which suggested that the antidiuretic activity of serum is related to the coagulation of the blood (Dicker and Ginsburg, 1950). With the development of a relatively simple, sensitive and precise procedure for the assay of the antidiuretic activity of plasma (Stein, Jinks and Mirsky, 1952), it became pertinent to re-examine the role of the adrenal glands and of the hypophysis in the secretion of antidiuretic substances (ADS) into the circulation.

METHODS

Male albino rats of the Carworth strain were used. The antidiuretic activity of the pooled plasma from 4 to 8 rats was assayed by the procedure described in a previous

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[3] U. S. Public Health Service Research Fellow of the National Institute of Mental Health.

report (Mirsky, Stein and Paulisch, 1953). Since Pitressin[4] was used as the standard in the bioassay, the antidiuretic activity of the plasma is expressed as equivalents of mU Pitressin per 100 ml.

Bilateral adrenalectomy was performed via the dorsal route under ether anesthesia. Except where otherwise stated, each rat was given an intraperitoneal injection of one mgm. cortisone immediately after the operation and thereafter permitted free access to 1% NaCl until 48 hours before any experimental procedure; the rats which were bled 18 hours after adrenalectomy were not given cortisone or NaCl. Two groups of hypophysectomized rats were utilized; one purchased from the Hormone Assay Laboratories of Chicago and the other operated upon in our laboratories via the parapharyngeal route. The hypophysectomized rats were maintained on a relatively high carbohydrate diet and permitted free access to water. After decapitation and bleeding, the operative site was carefully examined with a magnifying glass for evidences of hypophyseal remnants. The plasma from only such animals which appeared to be completely hypophysectomized was used. Subsequently, sections of the sella turcica were made in order to establish the completeness of the operation.[5]

RESULTS

Effect of Adrenalectomy

The antidiuretic activity of the plasma, expressed in terms of mU Pitressin per 100 ml., was determined in 18 hours, 7 days and 30 days after adrenalectomy. It is evident from Figure 1 that the immediate response to adrenalectomy was an increase in the antidiuretic activity of the plasma but that by 30 days the titer approached the values obtained in normal rats. The plasma ADS (mean $\pm$ S.E. per 100 ml.) of 97 normal rats (14 assays) used as controls was 18.4 ± 1.4 mU. The values obtained in 18 hours, 7 days and 30 days after adrenalectomy were 49.0 ± 8.3 mU for 39 rats (9 assays), 76.4 ± 9.2 mU for 56 rats (8 assays) and 28.0 ± 4.6 mU for 19 rats (4 assays), respectively. The differences between the plasma ADS of the normal controls and those of the 18-hour and 7-day adrenalectomized rats were statistically highly significant ($P < 0.001$), while the difference between the controls and the 30-day adrenalectomized rats was statistically less significant ($P = 0.01$–0.02).

The effect of treatment with cortisone on the antidiuretic activity of the plasma of adrenalectomized rats is illustrated in Figure 2. One group of 28 rats was given 2 mgm. cortisone intraperitoneally immediately after the operation, 1 mg. subcutaneously in 8 hours and 1 mg. subcutaneously in 16 hours and decapitated and bled in 18 hours. Another group of 32 rats was given 1 mg. cortisone intraperitoneally immediately after the operation and 2 mg. subcutaneously daily for 7 days. In both groups, cortisone prevented the increased antidiuretic activity that occurs in the plasma of

[4] We are indebted to Dr. D. A. McGinty of the Parke Davis Company for a supply of Pitressin which assayed at 40 U.S.P. Units per mg.

[5] We are indebted to Dr. Frank Dixon, Department of Pathology, University of Pittsburgh School of Medicine, for preparation and examination of the sections.

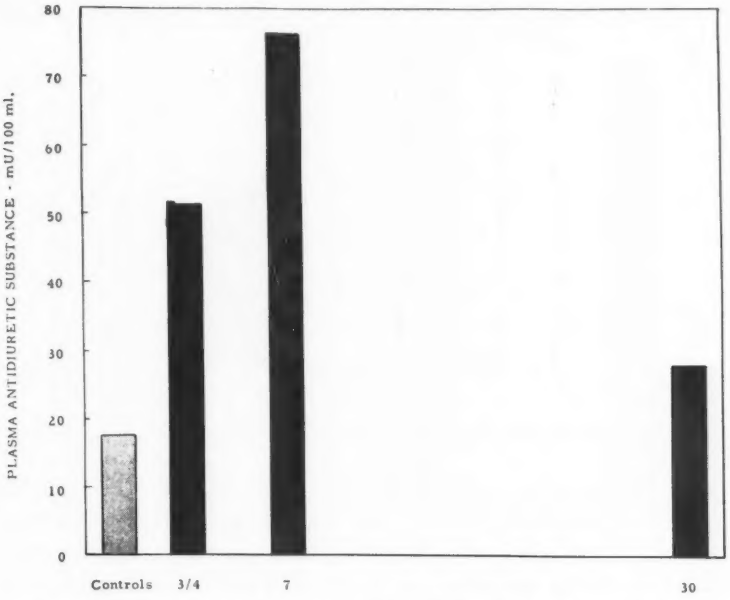

FIG. 1. Effect of adrenalectomy on the antidiuretic activity of the plasma.

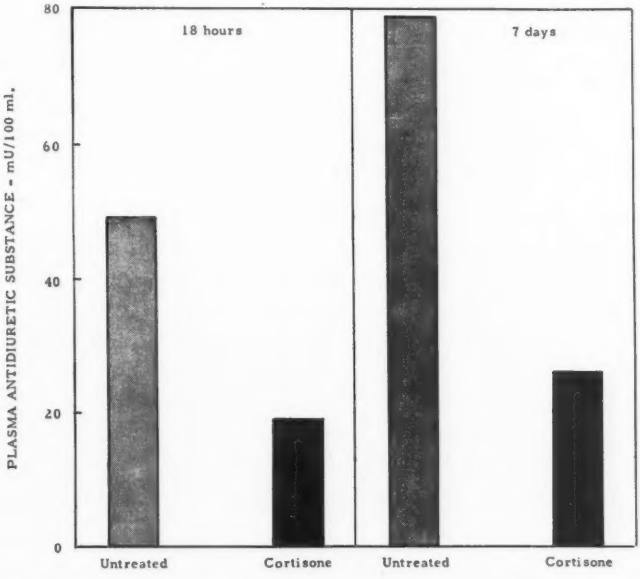

FIG. 2. Effect of treatment with cortisone on the antidiuretic activity of the plasma of adrenalectomized rats.

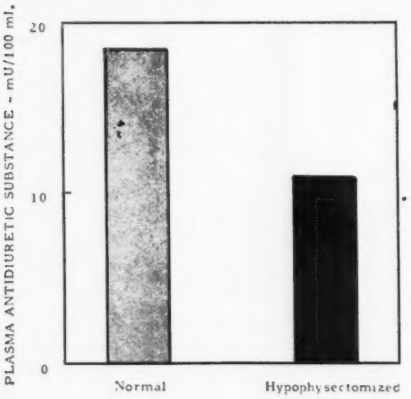

FIG. 3. Effect of hypophysectomy on the antidiuretic activity of the plasma.

both 18-hour and 7-day adrenalectomized rats. Whereas the antidiuretic activity of the plasma of the untreated 18-hour adrenalectomized rats was 49.0 ± 8.3 mU, and that of 7-day untreated adrenalectomized rats was 76.4 ± 9.2 mU, those of the cortisone treated groups were 19.2 ± 0.9 mU and 26.2 ± 3.5 mU respectively. The differences between the untreated and treated groups were statistically significant.

Effect of Hypophysectomy

The plasma antidiuretic activity of 27 rats (5 assays) was determined in from 5 to 10 days after hypophysectomy and found to be 10.9 ± 2.0 mU (Fig. 3). The difference between the values obtained in the normal and hypophysectomized rats was statistically significant ($P = 0.01$).

Effect of Adrenalectomy in Hypophysectomized Rats

Four days after hypophysectomy, one group of 29 rats was subjected to a bilateral adrenalectomy and another group of 16 rats to a sham adrenalectomy in that the adrenals were exposed and some peri-adrenal fat was removed. Both groups of animals were sacrificed 18 hours later and the plasma ADS concentration assayed. Figure 4 illustrates the observation that whereas sham adrenalectomy resulted in a small but statistically significant rise ($P = 0.01$–0.001) in the concentration of ADS in the plasma from 10.9 ± 2.0 mU to 22.6 ± 1.5 mU (3 assays), adrenalectomy resulted in a marked augmentation of the plasma ADS to 113.7 ± 17.1 mU (5 assays) ($P < 0.001$).

DISCUSSION

The increased antidiuretic activity of the plasma of untreated adrenalectomized rats, the inhibition of this increase by the administration of

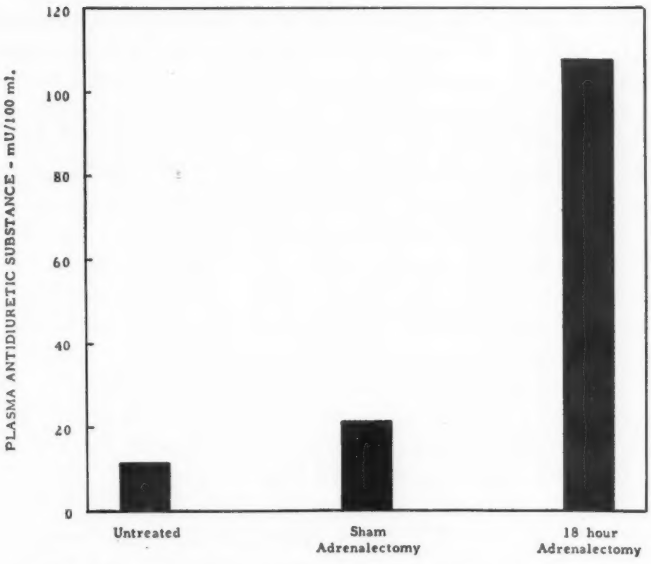

FIG. 4. Effect of adrenalectomy on the antidiuretic activity of the plasma of hypophysectomized rats.

cortisone, the relatively low antidiuretic activity of the plasma of adrenalectomized rats maintained with NaCl for 30 days, and the decrease in the antidiuretic activity of the plasma of hypophysectomized rats are essentially in agreement with the observation of Birnie *et al.* (1949, 1950). Such observations favor the hypothesis first proposed by Silvette and Britton (1938) and extended by others (Gaunt, Birnie and Eversole, 1949), that the adrenal cortex and the neurohypophysis operate as physiological antagonists in the regulation of the excretion of water. In opposition to this hypothesis, however, is the marked increase in the antidiuretic activity of the plasma from completely hypophysectomized rats after adrenalectomy.

The mechanism whereby adrenalectomy results in an increase in the antidiuretic activity of the plasma is unknown. The increase may be due to a diminution in the rate at which the antidiuretic hormone is removed from the circulation since Birnie (1952, 1953) demonstrated a decrease in the *in vitro* rate of vasopressin inactivation by extracts of livers from adrenalectomized rats. This observation does not preclude the possibility that an increase in the secretion of ADH into the circulation is responsible for the augmented activity of the plasma. Such an increase may be secondary to the changes associated with adrenal insufficiency which, like other nonspecific noxious systemic stimuli, may activate the secretion of ADS into the circulation (Mirsky, Stein and Paulisch, 1953).

Complete hypophysectomy results in a significant diminution in the antidiuretic activity of the plasma. Consequently, it could be concluded that ablation of the neurohypophysis eliminates the major source of antidiuretic activity. It contradiction, however, is the relatively rapid and marked augmentation of the antidiuretic activity of the plasma which occurs after ablation of the adrenal glands from the previously hypophysectomized rat. This response to adrenalectomy would appear quite paradoxical were it not that hypophysectomy produces no significant histological or histochemical change in the zona glomerulosa of the adrenal cortex of the rat (Deane and Greep, 1946). It may be postulated, therefore, that the corticosteroids produced by the zona glomerulosa of the hypophysectomized rat prevent the changes which occur in the completely adrenalectomized animal. After removal of the adrenals, however, the hypophysectomized rat develops a severe adrenal insufficiency. This insufficiency, like any other noxious stimulus, may activate the release of antidiuretic hormone from some extra-hypophyseal site. That the hypothalamus may be the extra-hypophyseal source of ADH is suggested by many studies which reveal that the supraoptic and paraventricular nuclei secrete ADH, vasopressin and oxytocin (Scharrer and Scharrer, 1945; Bargmann and Scharrer, 1951; Hild and Zetler, 1953). Particularly pertinent to the present study is the observation that adrenalectomy not only results in a decrease in the diuretic response to hydration and an increase in the antidiuretic activity of the plasma but also in a decrease in the antidiuretic activity of hypothalamic extracts (Kovacs and Bachrach, 1951).

The mechanism whereby cortisone prevents the increase in the antidiuretic activity of the plasma of adrenalectomized rats is unknown. The inhibitory effect may be related to the prevention of adrenal cortical insufficiency and thereby the prevention of such responses as occur with exposure to non-specific noxious systemic stimuli. On the other hand, cortisone may be effective through some direct action on the secretory cells of the hypothalamus. Such a direct effect is suggested by the chromatolysis of the paraventricular and other nuclei of the hypothalamus that occurs in rats given ACTH or cortisone (Castor, Baker, Ingle and Li, 1951).

In accord with the observations reported herein is the demonstration that the excretion of a water load by the hypophysectomized rat is further delayed after adrenalectomy (Hofmann, 1952). An explanation is not available, however, for the paradox that whereas the antidiuretic activity of the plasma of the adrenalectomized rat is increased and that of the hypophysectomized rat is decreased, both preparations have an impaired ability to excrete a load of water. It is quite possible that the procedure of administering the water load per stomach tube is in itself so noxious (Nagareda

and Gaunt, 1951) that the secretion of ADH is activated in both adrenalectomized and hypophysectomized rats.

SUMMARY

The antidiuretic activity of the plasma of rats is increased markedly in 18 hours after adrenalectomy. The plasma of animals adrenalectomized seven days previously and maintained with 1% NaCl for five days has a still greater antidiuretic activity. The activity of the plasma from animals adrenalectomized 30 days previously and maintained with NaCl for 28 days approaches that of unoperated rats. The administration of cortisone prevents the increased activity noted in 18 hours and 7 days after adrenalectomy.

Complete hypophysectomy results in a significant diminution in the antidiuretic activity of the plasma. Subsequent adrenalectomy, however, produces a relatively rapid and marked augmentation in the activity of the plasma. Consequently, the major source of the antidiuretic activity must be some site other than the neurohypophysis.

The effect of adrenalectomy is attributed to a non-specific activation of the hypothalamic-hypophyseal system by the noxious changes associated with the insufficiency of the corticosteroids produced by the zona glomerulosa.

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EFFECT OF THE SODIUM-RETAINING FACTOR OF THE ADRENAL CORTEX UPON THE SERUM ELECTROLYTES OF ADRENALECTOMIZED DOGS[1,2]

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AFTER removal of the known active crystalline compounds from adrenal cortical extracts, there still remains behind in the mother liquor a highly potent, life-maintaining residue known as the amorphous fraction (Wintersteiner *et al.*, 1934; Kendal *et al.*, 1937; Mason 1939; Wells and Kendall 1940; Ingle and Kendall 1940; Pfiffner 1942; Eversole *et al.*, 1942; Kuizenga 1944; Ingle 1950; Thatcher and Hartman 1946). Until recently this material resisted all efforts to isolate the active ingredient in pure form, but following the introduction of a relatively simple method for accurately determining electrolyte excretion of rats (Tait *et al.*, 1952 and Grundy *et al.*, 1952), progress has been rapid and recently culminated in crystallization of the Na-retaining factor (Simpson *et al.*, 1953 and Mattox *et al.*, 1953), and preparation by others of highly purified but non-crystalline fractions (Knauff *et al.*, 1953).

The Na-retaining factor employed in this investigation was prepared from extract concentrates of beef and hog adrenals obtained by Dr. Kendall from Parke, Davis and Company of Detroit, Michigan. The purest fractions used were not crystalline, and gave positive Porter-Silber reactions. These reactions indicated that 40% of the total steroid content (determined by U. V. absorption) consisted of 17, 21-dihydroxy-20-ketosteroids. The dose levels reported for the assays represent total U. V. steroidal content, and hence a maximum of only 60% of the dosages given in the tables can be considered to be pure Na-retaining factor.

METHODS

The test animals were male, mongrel dogs which had been adrenalectomized for periods ranging from 1–4 years; each had been used numerous times in previous studies

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[1] The chemical fractionation and purification of the sodium-retaining factor from adrenal extracts was done by Dr. E. C. Kendall and co-workers at the Forrestal Research Center of Princeton University. We are indebted to these investigators for the material used in this study.

[2] Grateful acknowledgement is made to Sharp & Dohme, Inc., West Point, Pennsylvania, and to the National Science Foundation, Washington, D.C., for financial support.

on the effectiveness of steroid preparations for maintenance.

It is imperative in tests involving adrenal steroids that the Na and K content of the diet be kept constant since variations in the quantities of these ions will markedly influence the results. The total amount of Na the dog received each day including that contained in the food was 0.93 gm. and for K the figure was 0.94 gm. This is a low Na intake and adrenalectomized dogs maintained on a daily dose of 0.5 mg. DCA and fed a diet containing this amount of Na will eventually develop insufficiency symptoms unless given some additional salt. However, these animals are readily maintained with normal serum Na levels if injected with small doses of the Na-retaining factor.

During the test period, the dogs were kept at each dose level for 10 days, blood samples were taken and the amount of the fraction under study reduced by 50%. This procedure was continued until the failing dose was reached. All injections of the water-soluble material were given subcutaneously twice daily in divided doses. In this study we have relied upon alterations in body weight, mean arterial pressure, blood urea nitrogen and serum electrolytes to indicate the end point of the tests. The methods used for determining arterial pressure and blood constituents have been adequately described in earlier studies (Swingle, Barlow *et al.*, 1952 and Swingle, Collins *et al.*, 1952).

GENERAL SCHEME OF FRACTIONATION OF THE AMORPHOUS MATERIAL FOR THE NA-RETAINING FACTOR

Each ml. of cortical extract which served as starting material contained the hormones from 204 gm. of fresh adrenal gland. Preparation of the material rich in Na-retaining factor was based on three procedures. One, partition between water and benzene, with separation of "A" compounds soluble in water but not in benzene; separation of "B" compounds soluble in benzene but not in water; separation of "C" compounds soluble in both water and benzene. Two, removal of cortisone, hydrocortisone and other steroids by crystallization from chloroform solution at −20° C. from 1–10 days. The Na-retaining hormone remained in the mother liquor. The final procedure was fractionation of the mother liquor in a Craig counter-current distribution instrument. The activity of any sample was related to the presence of an unsaturated ketone. Absorption of light at 288 $\mu\mu$. in an aqueous solution was used as a measure of the amount of unsaturated ketone present.

TESTS OF MATERIAL FRACTIONATED BY REPEATED DISTRIBUTION BETWEEN WATER AND BENZENE (CRUDE AMORPHOUS FRACTION-SOLUTION 21)

This fraction was prepared by first distributing the extract concentrates 6 times between water and benzene and then treating the water-soluble material in the Craig instrument for 200 transfers. All of the tubes were then combined for testing in order to determine whether activity was destroyed in the Craig apparatus. Crystalline steroids could not be separated from a chloroform solution. The material was then distributed 4 more

TABLE 1. CHANGES IN ARTERIAL PRESSURE AND BLOOD CONSTITUENTS OF ADRENALECTOMIZED DOGS RECEIVING CRUDE AMORPHOUS FRACTION (SOLUTION 21)

Dog	Dose	Exper. period	Wt.	B.P.	Blood urea N	Hb	Hmct.	R.B.C.	Blood sugar	Serum Na	Serum Cl	Serum K
	μg.	days	kg.	mm. Hg	mg. %	gm. %	%	10^6 cu. mm.	mg. %	m.eq./l.	m.eq./l.	m.eq./l.
		Initial	12.6	104	19.3	10.0	28.0	—	80.0	143.3	116.0	4.5
	312.00	10	12.7	89	20.0	—	—	—	—	140.4	118.9	4.3
	156.0	10	12.7	72	23.7	10.4	27.9	5.3	80.5	144.6	119.3	4.4
1	78.00	10	12.0	66	25.5	11.0	29.0	5.7	68.0	147.3	118.9	4.3
	39.00	10	13.2	83	41.1	10.0	27.1	5.2	72.0	143.0	114.6	4.7
	19.50	10	13.4	64	52.2	11.8	32.5	5.6	77.0	142.6	110.7	5.8
		Initial	23.5	107	19.7	10.8	28.4	5.6	82.5	146.0	116.5	4.0
	312.00	10	22.9	88	18.3	9.4	27.8	5.3	62.0	145.2	118.1	4.8
	156.00	10	23.1	99	23.0	9.5	30.0	5.8	79.5	147.6	112.9	4.6
2	78.00	10	22.3	89	28.7	12.2	34.1	6.2	76.5	140.0	108.3	4.7
	39.00	10	22.6	74	28.8	11.5	34.0	6.5	79.0	141.6	106.4	5.8
	19.50	10	22.2	74	52.2	12.3	35.1	7.1	80.5	140.0	107.8	7.5

times between water and benzene and afforded a "C" solution of high potency.

Table 1 gives the essential data obtained from study of 2 representative dogs of a total of 6 animals tested on the fraction. The weight of the steroid material in μg. as determined by ultra-violet is given in the table. The dose of steroid upon which five of the dogs developed adrenal insufficiency was 19.50 μg. but one dog developed adrenal insufficiency at 9.75 μg. The minimum maintenance dose was considerably higher, depending upon whether one selected as a test criterion, changes in mean arterial pressure or blood urea nitrogen.

It is obvious from the table that serum Na and Cl changes furnished no clue of impending adrenal insufficiency, and the serum K alterations were too inconstant to be relied upon. In 5 of the 6 dogs tested, the first sign of insufficient dosage was a slow decline in mean arterial pressure followed by increases in blood urea nitrogen. The serum K of dog 6 rose to 8 m.eq./l. and the test was discontinued. The arterial pressure did not decline appreciably during the 35 day interval. The animal is exceptional in this respect. The most impressive feature of the prolonged tests concerned the serum electrolytes. The serum Na and Cl remained within normal limits even when the dog exhibited severe symptoms of adrenal insufficiency. However, the level of the serum K was quite variable. In some animals (No. 1 Table 1) it either remained unchanged or was slightly elevated during the 50 days of the test, whereas in other dogs (e.g., No. 2) it rose markedly and occasionally attained levels which precipitated serious cardiac episodes due to hyperpotassemia. Apparently the behavior of serum K in adrenalectomized dogs treated for long periods with the Na-retaining factor, is identical with that observed when cortisone is the sole steroid used for maintenance (Swingle, Barlow *et al.*, 1952). The cation slowly accumulates until levels are obtained which may be incompatible with life.

FRACTIONATION OF AMORPHOUS MATERIAL BY MEANS OF THE CRAIG COUNTER-CURRENT INSTRUMENT (SOLUTION 37)

This fraction was prepared as follows: The crude amorphous material was separated from 1000 pounds of beef adrenals (see solution 21) and then put through the Craig for 200 transfers. Tubes 115–200 were discarded as inactive. The remaining tubes, 1–114 containing aqueous and benezene layers, were divided into 4 groups and each group independently concentrated and then extracted with chloroform. This latter solution was concentrated and placed at −20° C. for several days. The resulting crystals (cortisone and hydrocortisone) were removed by filtration and the filtrate then taken into water. These aqueous solutions from the various groups were recombined and this combination was subjected to the 200-tube Craig instrument for 300 transfers. The benzene portion of tubes 200–300 contained only 3 mg. of steroid (U. V.) and were discarded. The aqueous and benzene layers were divided into 3 groups and each treated with chloroform as previously described, only the group of tubes 131–200 afforded crystals. They were filtered off and the filtrate combined with the solutions of the other two groups of tubes. This solution was taken into water and the chloroform removed. The remaining solution is referred to as 37 and contained but very small amounts of cortisone and hydrocortisone. It was a non-crystalline but highly purified fraction. Pertinent data regarding physiological activity of the fraction are given in Table 2. Only two animals were used for the study which extended for 50 days, however, since the results were similar in both cases it was not considered necessary to make further tests. The starting dose of steroid material was 100 μg. as determined by U. V., and the daily amount which led to development of severe adrenal insufficiency was 6.25 μg. administered for 10 days. As in all of the tests, a decline in the mean arterial pressure and rise in blood urea nitrogen occurred. The serum Na revealed no noteworthy changes but remained

TABLE 2. BLOOD CHANGES IN ADRENALECTOMIZED DOGS RECEIVING A PURIFIED FRACTION OF THE Na-RETAINING FACTOR (SOLUTION 37)

Dog	Dose/ dog/day	Exper. period	Wt.	B.P.	Blood urea N	Hb	Hmct.	R.B.C.	Blood sugar	Serum Na	Serum Cl	Serum K
	μg.	days	kg.	mm. Hg	mg. %	gm. %	%	10^6 cu. mm.	mg. %	m.eq./l.	m.eq./l.	m.eq./l.
3		Initial	15.8	103	19.5	10.7	29.6	5.2	77.0	142.5	113.1	3.9
	100.00	10	15.4	89	23.4	11.2	29.7	5.2	76.0	140.5	109.9	4.3
	50.00	10	15.6	80	34.3	10.6	28.8	5.2	77.0	140.0	110.7	4.4
	25.00	10	15.2	74	31.2	9.8	26.8	5.0	77.5	141.3	111.1	4.3
	12.50	10	14.7	72	32.5	10.0	30.4	5.2	78.5	140.0	109.0	5.5
	6.25	10	14.5	60	34.3	11.8	36.4	5.6	77.5	138.7	106.5	6.5
4		Initial	14.5	100	22.0	10.8	30.3	6.1	77.5	143.4	114.0	4.4
	100.00	10	14.3	95	25.6	12.8	38.8	6.7	73.0	138.7	110.1	4.6
	50.00	10	14.4	92	28.3	11.2	30.9	6.2	75.0	138.5	110.4	5.1
	25.00	10	14.1	94	25.5	13.5	32.1	6.3	80.5	139.0	111.7	5.2
	12.50	10	14.0	78	32.7	12.0	36.1	7.1	79.5	138.3	109.4	6.6
	6.25	10	13.8	66	49.2	9.6	29.0	6.5	71.5	136.0	107.3	8.2

within the lower range of normal even when symptoms were present, but the serum Cl slowly fell 6.6 m.eq./l. The serum K of dog 4, Table 2, was strikingly elevated at the close of the test and was within the range where cardiac disturbances became evident. Both dogs exhibited unmistakable symptoms characteristic of insufficiency when the test was terminated. The animals were restored to normal health by administration of DCG and saline-glucose solution by vein over a period of several days.

FRACTIONATION OF EXTRACT CONCENTRATES PREPARED FROM HOG GLANDS. (SOLUTION 39)

The crude amorphous fraction was prepared in a manner identical with that of solution 21. This fraction was subjected to 300 transfers in the 200-tube Craig counter-current instrument. The tubes were grouped into 4 sets and crystals of cortisone and hydrocortisone removed from each group independently as outlined previously for solution 37. The filtrates were then taken back into water. The activity of tubes 74–94 was found to approximate that of solution 37, therefore, this was used as a representative sample of the Na-retaining factor obtained from hog glands.

Tests of the material indicated that the potency of fractions prepared from hog glands did not differ materially from that obtained from beef adrenals (Table 3). The failing dose as listed in the table was 3.125 μg., however, since the animal received this minute amount but one day before the blood pressure fell to 38 mm. Hg. the daily quantity of steroid material which should be regarded as the failing dose was 6.25 μg. This figure is identical with that obtained from tests of beef material (Table 2).

The maintenance of normal levels of serum Na and Cl despite the low dosage used is surprising but the data are in agreement with those recorded for tests on other fractions containing potent Na-retaining material and also with data recorded by others using pure, crystalline Na-factor (Simpson *et al.*, 1953 and Mattox *et al.*, 1953). The serum K seems to be much less affected by this factor since it exhibits a highly variable behavior and may be only moderately elevated or it may be retained until the serum concen-

TABLE 3. TEST OF A PURIFIED FRACTION OF THE Na-RETAINING FACTOR PREPARED FROM HOG GLANDS (SOLUTION 39)

Dog	Dose/ dog/day	Exper. period	Wt.	B.P.	Blood urea N	Hb	Hmct.	R.B.C.	Blood sugar	Serum Na	Serum Cl	Serum K
	μg.	days	kg.	mm. Hg	mg. %	gm. %	%	10^6 cu. mm.	mg. %	m.eq./l.	m.eq./l.	m.eq./l.
		Initial	10.4	98	14.8	10.6	31.2	5.2	76.0	140.0	113.9	4.2
	100.00	10	10.2	89	15.0	11.6	32.9	5.4	81.5	139.3	110.4	4.1
	50.00	10	10.0	76	18.9	10.0	28.0	5.3	77.0	137.7	109.8	4.4
5	25.00	10	9.9	73	21.5	9.4	30.7	5.4	82.5	139.6	110.0	5.0
	12.50	10	9.9	72	17.5	10.4	28.3	5.7	76.5	136.3	109.4	5.0
	6.25	10	9.9	74	25.8	12.4	32.2	5.8	79.0	137.8	111.0	5.9
	3.125	1	9.9	38	29.9	15.1	40.0	8.3	66.0	137.7	110.9	6.1

tration becomes extremely high. Dog 5, Table 3 is an excellent example of the termination of a test by circulatory failure. As revealed in the table, noteworthy changes in the serum electrolytes were absent, the blood urea nitrogen had increased 100% above the initial value, the blood glucose was moderately depressed and hemoconcentration was present, but none of these changes were of sufficient magnitude to induce the severe symptoms observed. These were due primarily to vasomotor failure and peripheral vascular collapse. Thus, it is apparent that daily doses of the Na-retaining factor which are sufficient to maintain a normal level of Na in the serum may be unable to prevent the slow decline in arterial pressure characteristic of adrenal insufficiency.

DISCUSSION

The test employed in these experiments for determining the potency of various fractions of the Na-retaining factor is slow, requiring 40–50 days for completion and hence, presumably may allow sufficient time to permit the dog to make some adjustment to each successive dose level. However, if a sufficient number of criteria are considered for judging the onset of the early symptoms of adrenal insufficiency, a reasonable degree of accuracy is obtainable. The tests when conducted in the manner described are readily reproducible but do require a great deal of time. Valuable physiological data can be collected in these prolonged observations which are not readily obtainable when more rapid tests are employed.

It is obvious from the tabular data presented, that alterations in levels of serum electrolytes such as fall in Na and Cl and rise in K, cannot be relied upon for determining the end point of a test involving the Na-retaining factor. It has been observed repeatedly that dogs receiving such material may exhibit the classical symptoms of adrenal insufficiency and appear practically moribund, yet have serum Na ranging from 138–144 m.eq./l. and serum Cl varying anywhere from 100–114 m.eq./l. The serum K may be only slightly elevated or may be extremely high with concentrations of 8, 9, and 10 m.eq./l. associated with severe cardiac disturbances and prostration.

The sharp increase in serum K during the test may occasionally afford the only clue to impending collapse; we have lost several animals through failure to closely follow the slow increase in the level of this electrolyte. It is not unusual to observe animals with extremely high levels of serum K but with normal levels of Na. It would seem, therefore, that the Na-retaining factor is much more efficient in regulating the renal excretion of Na than it is of controlling the elimination of K. This has also been observed by Farrell and Richards (1953), using a potent Na-retaining substance isolated from adrenal venous blood of the dog.

The adrenalectomized dog fed a diet low in Na such as used in these experiments, requires a surprising quantity of cortisone for maintenance. Swingle, Collins *et al.*, (1952), found that such dogs must receive at least 1.86 mg./Kg./day of the microcrystalline preparation in order to keep the serum electrolyte pattern normal. Hence, it is apparent that the minute amounts of cortisone and hydrocortisone contaminating the daily dose of the Na-retaining factor used in this study could not be responsible for the marked potency of the material. Studies have been made by the writers, on water-soluble cortisone prepared for us by Dr. E. C. Kendall. The solution contained 1 mg./cc. of the free alcohol; this amount administered twice daily proved ineffective and failed to maintain the animals free from symptoms of adrenal insufficiency. Further experimentation using 2.5 mg. dog/day of both the free alcohol of cortisone and hydrocortisone, dissolved 95% ethanol and diluted dropwise with warm water to a 10% solution and injected twice daily in divided doses, also failed to maintain a normal serum electrolyte pattern or keep the dog symptom-free.

The slow but steady decline in the mean arterial pressure of the dogs receiving the Na-retaining factor, strongly suggests that this substance lacks an important function characteristic of cortisone and hydrocortisone. Although these steroids fail to maintain the normal serum electrolyte pattern except when given in very large doses, they do maintain the arterial pressure despite the sharply lowered serum Na and Cl. According to Knauff, Nielson and Haines (1953), the sodium factor lacks two other functions usually associated with 11-oxysteroids viz., ability to induce: (1) glycogen deposition in starved adrenalectomized rats; and (2) a fall in the eosinophils in these rodents.

SUMMARY

By using suitable fractionation methods, refined but non-crystalline water-soluble steroid material (U.V.) was removed from the crude amorphous fraction derived from extract concentrates of beef and hog adrenals. Tests on adrenalectomized dogs showed the material to be many times more potent than DCA in maintaining normal serum levels of Na. However, it is much less effective in regulating serum K than it is in regulating serum Na since many of the test animals developed hyperpotassemia accompanied by severe cardiac disturbances despite exhibition of normal serum Na levels, The Na-retaining factor as employed in the dosage used in these experiments, failed to sustain the arterial pressure which slowly (over a period of weeks) declined to levels necessitating restorative measures. The serum Na in numerous tests remained unchanged from its initial value even when the dogs were displaying severe symptoms of adrenal insufficiency.

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NOTES AND COMMENTS

A TECHNIQUE FOR KILLING ANIMALS TO OBTAIN TISSUES FOR STUDIES ON STRESS EFFECTS[1]

Biochemical studies on the effects of stress, such as the changes in adrenal ascorbic acid and cholesterol, necessitate the use of biological control samples obtained from unstressed animals. The difficulty arises in trying to get samples from an animal without

FIG. 1. Cages for shooting small animals. Dimensions: outside, 31×19×15 in., inside, 24×12×11 in., legs 5 in. high. Sides and bottom made of 2×4 timbers, outside jacket of galvanized sheet metal (not essential), bottom covered with galvanized tray with central drain hole, hinged lid of framed metal grill.

producing stress in the process. Obviously, the perfect control sample is unobtainable because it is impossible to avoid some disturbance of the animal in the sampling procedure. Nevertheless it is important to minimize the disturbance to permit detection of the greatest possible differences between samples obtained from stressed and unstressed animals.

Confronted with this problem, the authors considered and discarded the following

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modes of obtaining control samples because in each case the degree of physiological stress was considered too great: 1. operation under anesthesia, 2. killing by electrocution, decapitation, gassing, intravenous air injection or drug injection, and immediate removal of sample from the dead animal.

The method which was finally adopted provides a simple means of inflicting sudden death without fright or apprehension, touching or disturbing the animal. In work on the adrenal it has been possible to obtain glands and freeze them with solid carbon dioxide or in a bath cooled by liquid nitrogen for subsequent chemical study within a minute or two from the time that the animal was peacefully occupied in normal activities. The

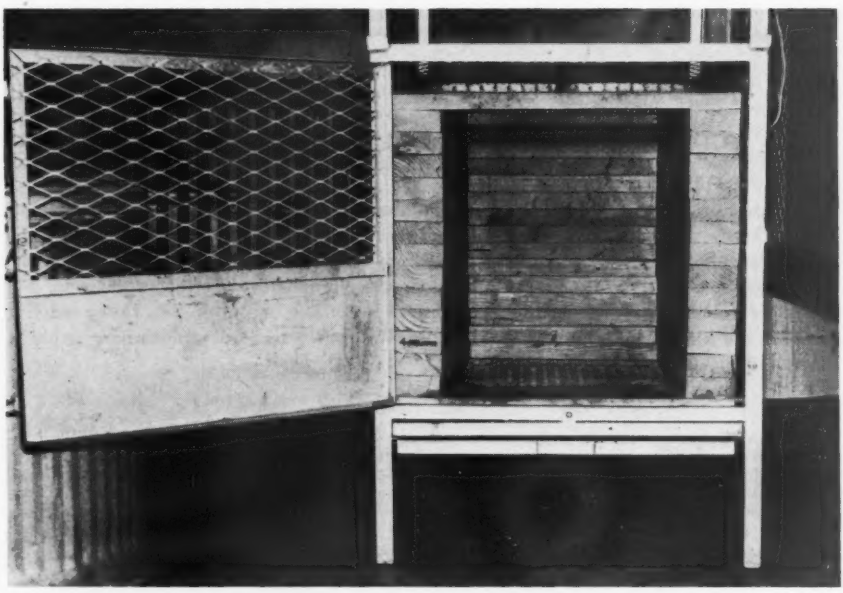

FIG. 2. Cage for shooting monkeys. Standard commercial cage lined with 2×4 timbers on 3 sides as shown.

limiting time factor is the speed with which the experimenter can remove the sample from the dead animal; the killing itself is almost instantaneous.

This result has been achieved by permitting the animal to live normally in a special cage for a length of time adequate for acclimatization to the surroundings and routine (usually 4–7 days was taken for monkeys), and then the animal is shot in the back or side of the head at a distance of several feet with a .22 caliber "long" hollow-point lead bullet fired from a rifle.

The "long" bullet is used to provide sufficient force to embed the lead in the special walls of the cage without ricochet. The hollow-point form provides a bullet which expands after first striking the head and destroys a greater portion of the brain than a solid bullet could. A rifle is preferable to a pistol because it permits accurate firing from a greater distance, and thus it is possible to remain far enough away from the animal to avoid disturbance before firing. A single shot rifle with a safety catch was used to

minimize chances of accident, and it was never loaded until immediately before shooting.

The cage used for small animals is illustrated in Plate 1, and that for larger animals (monkeys) in Plate 2. The metal grill on the cage doors has openings large enough to permit firing through them while the doors are closed; care in aiming is required to avoid hitting the metal of the grill. A grill with larger openings would no doubt be more convenient for many.

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GLOMERULAR FILTRATION RATE AFTER ADRENAL MEDULLECTOMY IN THE RAT

We have previously confirmed the observations of Stein and Wertheimer (1944) that the diuretic response to water loading in the rat is reduced by adrenal medullectomy (Dexter and Stoner, 1952). Our results indicated that this was not due to failure of cortical regeneration but to the loss of medullary tissue one effect of which was to prevent the immediate cortical response to the stress of water loading. However, the possibility that the operation reduced the glomerular filtration rate (GFR) was not investigated in detail and this has now been done.

METHODS

Male albino rats from the same source and fed on Edinburgh rat cube No. 86 (20% protein) were used. The operative techniques etc. have been given previously (Dexter and Stoner, 1952). The absence of the medulla was confirmed histologically in each case. Renal clearances were determined on rats fasted overnight and after they had been given three doses of 5.0 ml. water per 100 gm. body wt. by stomach tube at hourly intervals, and at a time when the difference in the diuretic response in the normal and medullectomized rats was apparent. Inulin clearance (C_{IN}) determinations were based on the methods of Dicker

TABLE 1. GLOMERULAR FILTERATION RATE IN ADRENAL MEDULLECTOMIZED AND ADRENALECTOMIZED RATS

	No. of rats	Body wt., gm.	C_{IN}, ml./100 gm.	C_{Cr}, bodywt./min.
Normal Controls	19	207 ± 23	0.33 ± 0.13	—
Normal Controls	24	200 ± 17	—	0.67 ± 0.10
Medullectomized				
33–38 days post op.*	17	217 ± 20	0.33 ± 0.10	—
37 days post op.*	12	204 ± 20	—	0.63 ± 0.12
9 days post op.†	7	192 ± 14	0.45 ± 0.23	—
Adrenalectomized				
2 days post op.‡	4	195 ± 11	0.04 ± 0.03	—

* Given 1% NaCl to drink for 1 wk. post op.
† Given 1% NaCl to drink for 4 days post op.
‡ Not maintained on 1% NaCl.

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and Heller (1945) and Friedman, Polley and Friedman (1947). Three to four ml. of a 2% solution of inulin in 0.9% NaCl were given intraperitoneally at the time of the third dose of water. The collection period began 50 min. later and lasted 10–20 min. At the end of the collection period the rats were killed by a blow on the head and bled from the chest. Inulin, in plasma and urine, was determined as described by Friedman *et al.* (1947). Exogenous creatinine clearances (C_{Cr}) were similarly determined on hydrated rats. Fifty mg. creatinine were given subcutaneously at the time of the third dose of water. A high plasma creatinine level (0.8 mg./ml.) was used to minimize any interference from the possible tubular excretion of creatinine (Fingl, 1952). Creatinine in plasma and urine was determined by the method of King (1951). The clearances are expressed as ml. plasma per 100 gm. body wt. per min. and the values given are the means ± standard deviation.

RESULTS

In the normal rats the C_{IN} values were in the same range as those given by Dicker and Heller (1945). The values for the exogenous C_{Cr} in the normal rats were higher than those for C_{IN}, in accordance with the findings of previous authors, but were somewhat lower than those given by Lippman (1947) and closer to those found by Friedman (1947). However, it is quite clear that neither the C_{IN} nor the C_{Cr} in the medullectomized rat is significantly different from the control values even as early as 9 days after the operation when the combined adrenal weight is only 46% of the normal level and the cortex about a third of its normal width. Consequently, in adult medullectomized rats, as in normal rats, with a high protein intake (Dicker, 1949) the GFR is independent of the rate of urine flow. The low values for C_{IN} in the adrenalectomized rats not maintained on NaCl confirms the work of others (reviewed by Gaunt, Birnie and Eversole, 1949) and illustrates the great reduction in GFR in adrenal cortical deficiency.

CONCLUSION

The glomerular filtration rate is not affected by adrenal medullectomy in the rat.

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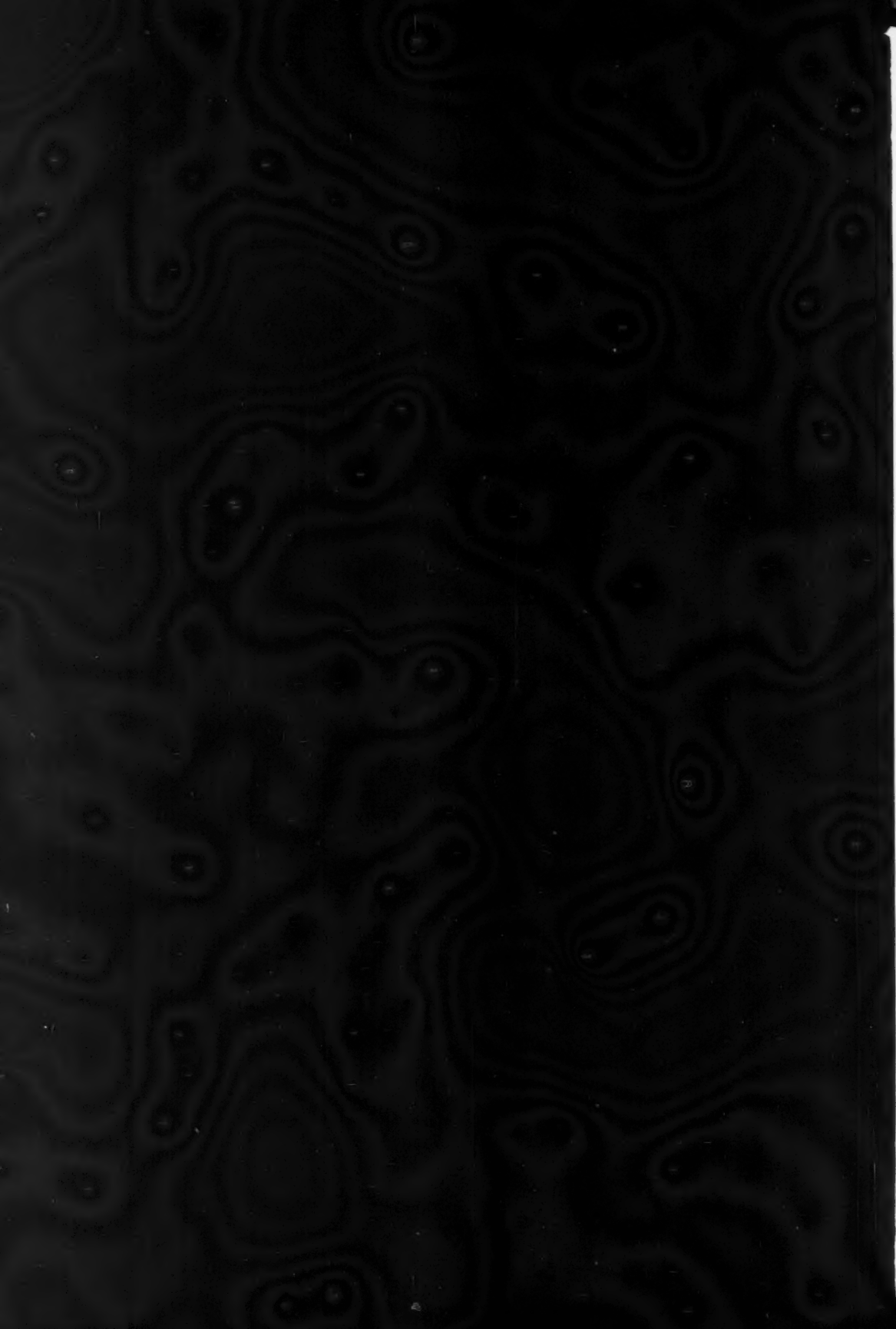

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